THE IDENTIFICATION OF ANTI-IDIOTYPIC ANTIBODY DURING AN IMMUNE RESPONSE IN DOGS

BY

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This dissertation is dedicated to my wife Nancy.
Without her love and support (and typing), it
would never have been.

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This investigation commensed with the development of an animal model to study the synthesis of IgE antibody. Repeated immunizations with a haptenated parasite extract (dinitrophenol coupled ascaris) in young dogs resulted in the production of anti-DNP antibody of the IgE, IgG and IgM class.

Although attempts to regulate this anti-hapten antibody response by administration of autologous anti-DNP antibody were unsuccessful, such therapy did result in the production of anti-idiotypic antibodies. These anti-idiotypic antibodies were demonstrable using mouse hybridoma-derived anti-DNP antibodies.

This antibody was shown to be anti-idiotypic rather than an internal image of antigen because it bound to only two of four monoclonal anti-DNP antibodies and failed to inhibit the id anti-id interaction with hapten. Anti-idiotypic antibodies were detected during the immunization schedule in three of five dogs using autologous anti-DNP $F(ab)'_2$ fragments as the source of idiotypes.

The anti-idiotypic antibodies identified using the mouse monoclonal antibody were the result of the immunization procedure and did not appear to be physiologically relevant to regulation of the immune response. On the other hand, the anti-idiotypic antibodies identified with the autologous source of idiotypes appear to be produced during the DNP-ASC immune response and were detected before autologous antibody immunization. The antigens that induced the anti-idiotypic response appeared to be, in this case, the idiotypes on the anti-DNP antibody that were produced from the DNP-ASC immunization.

CHAPTER ONE
INTRODUCTION

Allergic diseases of the immediate type are very important pathologic disorders in both man and dogs. Clinical signs are initiated by an interaction of antigen and IgE antibody with resultant mediator release from mast cells and basophils. In man and dogs, allergic reactions cause considerable morbidity and can be fatal (1,2). Anaphylaxis from a bee sting is a classic example for both species.

Atopy in man is an inherited disease which is associated with an antigen specific IgE response against environmental allergens. This disease is expressed clinically as asthma, hay fever, atopic dermatitis or any combination of these three (3). The dog is an excellent experimental animal model to study IgE mediated hypersensitivity for atopic diseases of man because of the similarity of the allergic reaction in both species (1,2,4). Canine IgE shares many physicochemical properties with human IgE (5,6,7). Dogs like man, develop spontaneous disease associated with increased synthesis of IgE antibody (3,4) and in dogs the disease is also familial (4).

There are a number of unique features of IgE antibody synthesis. Firstly, IgE circulates in very small amounts as compared to other antibody classes. In man, the serum level of this antibody is about 1/10,000 the level of serum IgG (3), and in dogs serum IgE is about 1/100 the level of serum IgG (7). Serum IgE levels of internally parasitized people and dogs are elevated as compared to non-parasitized individuals (7,8,9). The higher IgE level in dogs is felt to be the result of a greater parasite burden in this species (7).

Secondly, IgE is produced predominantly locally by lymph nodes in the respiratory and gastrointestinal tracts as well as in regional lymph nodes (10). These observations have led to the suggestion that this immunoglobulin is important in host defense of mucosal surfaces and particularly against parasites. Furthermore, IgE has been shown to participate in parasite killing through antibody dependent cell mediated cytotoxicity (11). Thirdly, the antigens that stimulate IgE antibody are usually very complex and heterogenous substances such as allergens or parasites and their extracts. When an animal is exposed to these antigens, the antibody response usually includes high titer IgE antibody whereas bacteria and viruses usually do not induce IgE antibody in spite of being very immunogenic (12).

The induction of IgE antibody experimentally requires special conditions. For example, high doses of antigen and strong adjuvants such as complete Freund's adjuvant are unfavorable to the development of an IgE response whereas low doses of antigen in an adjuvant such as aluminum hydroxide tend to favor IgE production (13). Furthermore, if haptens are coupled to parasite extracts, high titer anti-hapten IgE antibody responses will frequently develop. However, if the same hapten is coupled to a T-independent antigen or a different T-dependent carrier there is usually no IgE response (13,14). This suggests that IgE production is dependent on both the carrier and T-cells. The reason why parasites and their extracts are efficient inducers of IgE antibody is not fully understood. It is known that this enhancing effect is modulated through factors produced by T cells. Ishizaka's group (15) have shown that T cells derived from (N. brasiliensis) parasitized rats produce an IgE-potentiating factor which selectively potentiates a non-specific IgE antibody response. This factor has affinity for IgE, binds to IgE-bearing B cells through surface IgE and enhances the differentiation of these cells into IgE forming cells. A factor with similar properties has been produced from T cells obtained from patients with hyper-IgE syndrome, suggesting that the regulatory factors

and pathways for enhancing IgE antibody production for parasites and IgE in general might be similar (16).

A number of approaches have been used in an attempt to control allergic disease. Avoidance of the antigen is one approach, but this is rarely possible. Drugs that inhibit mediator release or control the effects of that release are also employed, but they have side effects and often require continual therapy. However, the ideal approach would be to regulate the production of the unwanted IgE antibody.

The mechanisms used to regulate IgE antibody responses involve either the inactivation of B cell precursors or the manipulation of T cell populations. To this end, primary and ongoing antibody responses, including IgE antibody, have been suppressed in mice by antigen coupled to non-immunogenic carriers such as d-glutamine-d-lysine (dGL) or polyvinyl alcohol (17,18). Both of these carriers inactivate hapten-specific B cells and can induce hapten-specific suppressor T cells. Moreover, when dGL is coupled to proteins rather than haptens, the resultant suppression in mice is isotype specific (i.e. suppresses IgE alone) (19). Unfortunately, there are no published results of the use of this compound in dogs or man.

Hyposensitization has also been used in an effort to control allergies in both man and dogs (20-22). The mechanism by which it works is not clear. It is known that

IgG antibody can have a role in regulating allergic symptoms (22). An IgG response can be induced by administration of allergen either by the normal route of exposure or by a route other than for normal exposure (i.e. subcutaneous versus inhalation). This IgG antibody presumably completes the allergen-IgE antibody interactions (21). However, this therapy is not without side effects (20). Furthermore, only about 65 per cent of patients treated with hyposensitization have clinical improvement (20).

An alternate type of hyposensitization involves modifying the allergen, usually by mild denaturation. Studies in mice with urea denatured ragweed showed that such treatment reduced allergenicity while maintaining immunogenicity of the allergen. If large doses of urea denatured ragweed were given to mice previously sensitized to unmodified ragweed, such therapy resulted in antigen-specific T suppressor cell induction without the development of anaphylaxis (12). These cells suppressed the anti-ragweed IgE response. A controlled study is underway to determine if this form of immunotherapy is any more effective in controlling allergic symptoms than conventional hyposensitization.

Another approach is to regulate the response with products of the immune system. Smith (23), in 1909, was the first person to recognize that antibody could suppress the

development of an immune response. In these experiments he showed that certain mixtures of diphtheria toxin and antitoxin could be very immunogenic in guinea pigs, but if there was a large excess of antitoxin, the immunized guinea pig would fail to mount an immune response against the toxin. Numerous studies in the 1950's and 1960's verified this observation and also demonstrated that the isotype, amount, affinity and time of administration were important variables in determining the degree of suppression that passive antibody had on the immune response (reviewed in 24). For example, IgG antibody given after antigenic exposure was more effective in inducing antibody suppression than IgM antibody. Further, the suppressed state was longer lived using IgG than IgM antibody. An interesting report by Chan and Sinclair (25) stated that the administration of anti-SRBC antibody given to mice after antigenic challenge led to a suppression of this response and this tolerant state could be transferred from one mouse to another with T-cells from the tolerized mouse. They suggested that the regulatory action of antibody operated through some sort of "induced pathway or secondary immune response" (25 p. 977).

In the early 1970's it was likewise shown that IgE antibody could be regulated by passively administered antibody (26-28). Rabbits were immunized to produce high titer IgE antibody and were given passive antibody 24 hours

after antigenic challenge. A complete inhibition of the passive cutaneous anaphylaxis titer and a marked decrease in the hemagglutination titer of these rabbits resulted as compared to controls (26). It was shown by Tada and Okumura (27) that, in the rat, the administration of anti-DNP ascaris antibody resulted in marked suppression of a preexisting IgE antibody response and this suppression was maintained for an extended period of time. This was in contrast to studies in the mouse in which administration of anti-ovalbumin IgG had little effect on the preexisting anti-ovalbumin IgE response (28). These differences were explained as species variation. Alternatively, they may be due to the difference in the antigenic system employed.

One explanation for the mechanism of regulation by passive antibody is that the administration of this antibody acted as an antigen and stimulated an anti-antibody response. Lahss et al. (29) were the first to show that some anti-antibodies would bind to structures on antibody close to or within the antigen combining site. These determinants have been named idiotypes (id) and the immune response directed to them is termed an anti-idiotypic (anti-id) response. In 1974, Jerne (30) proposed his network hypothesis of antibody regulation. The basic premise of this theory is that the immune system is regulated by a network of interactions between id and anti-id. A number of

assumptions are crucial premises to this theory. Firstly, most idiotypes exist at a level too low to induce tolerance. Thus, antigenic stimulation and expansion of these id will stimulate the production of a reciprocal set of anti-id. The id is then regulated directly by the anti-id, indirectly by the anti-id on T-cellsor or by anti-id acting on T-cells. As the concentration of anti-id reaches some critical threshold, a second anti-id response develops which is specific for the id of the anti-id. This anti-id would, therefore, be an anti-(anti-id) and would then stimulate a fourth response and so on, thereby resulting in an inter-related network of regulation between antibody molecules. Jerne also stated that id determinants can be present not only on antibody molecules of one specificity, but may be present on unrelated antibody molecules. Thus, antibody against antigen x might share some ids with antibody against antigen y. Lastly, although anti-id usually suppresses the corresponding id, it can be stimulating for the id as well. The anti-id would be expected to have a three dimensional structure similar or identical to the specific antigenic determinant. This type of anti-id is termed an internal image of antigen.

The characteristics of idiotypes of antibody molecules have been described (31-35). In many instances, idiotypes are located in or very near to the antigen binding site.

This has been demonstrated by hapten inhibition studies. Brient and Nisonoff (31) induced anti-p-azobenzoate antibodies in rabbits. These antibodies were purified and injected into allotypically matched rabbits and the resultant antiserum bound to determinants present on some rabbit anti-p-azobenzoate antibodies. They then studied the effects that adding increasing concentration of hapten would have on the reaction between radiolabelled anti-azobenzoate antibodies and the anti-idiotypic antiserum. They found that the binding affinities of the benzoate derivatives correlated closely with their ability to inhibit the antibody/anti-id interaction. In many other studies (32-34), anti-id was induced in animals immunized with an anti-hapten antibody. This anti-id was purified from the sera by initial adsorption to an affinity column having the immunizing antibody bound to it and was then eluted with the appropriate hapten. This purification process then would select for anti-idiotypic antibodies which were directed to those idiotypic determinants very close to or within the antigen binding site and it would be expected that hapten could inhibit the id/anti-id interaction.

On the other hand, it is not always possible for hapten to inhibit id/anti-id. For example, Sher and Cohn (35) showed that there was variation in the ability of hapten to inhibit id/anti-id interaction. Hapten was not able to

inhibit the interaction by 100 percent, maximum inhibition was only 68 percent (35). The most extreme example in which hapten cannot inhibit id/anti-id interactions are in those studies in which cross reactive ids are present on antibody molecules of widely different specificity. For example, Eichmann et al. (36) showed that one half of the A5A id producing clones in A/J mice immunized with a streptococcal carbohydrate lacked the ability to bind this antigen. Obviously then, antigen would not be expected to inhibit this id-anti-id interaction. In other studies, Bona et al. (37) showed that not all the id positive antibody following immunization with inulin could be removed with an inulin immunoabsorbent. In these experiments, the anti-inulin antibody produced following antigenic stimulation bears a predominant id. However, some immunoglobulin following antigenic stimulation had this id but lacked specificity for inulin. These experiments therefore suggest that some mechanism exists naturally in which id positive clones of immunoglobulin producing cells are expanded following antigen stimulation but that not all the id positive immunoglobulin is specific for the immunizing antigen. These experiments clearly show that although id/anti-id can usually be hapten inhibited, this property is not a requirement for an antibody to be anti-idiotypic.

Identical ids have been found irrespective of the isotype of the antibody. The mechanism by which IgE and IgG antibody can have identical idiotypes relates to the gene rearrangement that occurs during differential expression of heavy chain genes (38). As a single clone of cells goes through isotypic shift, a single variable region of the genes which includes the idiotype, will become linked to various heavy chain gene fragments. A single cell will differentiate into plasma cells which express different heavy chain genes but the same variable gene sequence (39). Therefore, it is possible for ids to be shared between antibodies of the same binding ability irrespective of the isotype. This implies that regulation of IgG antibody by anti-id networks may also result in IgE antibody regulation.

Idiotypic determinants are usually defined serologically. There are a number of different ways to produce anti-id (reviewed in 40-42). Anti-id can be produced across the species barrier, within the same species, within the same strain, or more importantly, even within the same individual that produced the id. Anti-id have been used to determine if the id of the antibody molecule may have a function other than to bind antigen. This has been done by examining what functional significance the presence of anti-id had on the corresponding id <u>in vivo</u>.

There are numerous reports that have shown that the passive administration of anti-id or the active induction of anti-id results in the suppression of the corresponding id (reviewed in 40-46). This modulation acts directly on B-cells or indirectly through T-cells. For example, in a B-cell tumor model, Balb/c mice immunized with MOPC 315 myeloma protein produced antibody with specificity for the id of MOPC 315. Subsequently these mice were injected with a MOCP 315 bearing plasmacytoma and the tumor growth was inhibited. It has also been shown that the immunization of MOPC 315 protein also induces idiotype specific T-suppressor cells that inhibit the MOPC 315 tumors secretion in vivo (47). Cosenza and Kohler (48) demonstrated that anti-id can act as an anti-antigen receptor antibody and specificially inhibit the induction of a primary immune response. In other studies by this same group, anti-id, which was specific for anti-phosyphorylcholine (PC) antibody, significantly inhibited anti-PC plaque forming cells to a degree similar to the inhibition seen with antigen (49).

These studies show that experimentally, the administration of anti-id or immunization with id to induce anti-id can result in id suppression. However, if anti-id regulates id during a normal immune response, auto-anti-id should be part of the response.

A number of studies have shown the presence of auto-anti-id during a normal immune response to an antigen (50-57). Bankert and Pressman (50) showed that an antibody with auto-anti-id activity could be detected in rabbits during primary and secondary immune response to both sheep red blood cells and to the hapten, 3-iodo-4-hydroxy-5-nitrophenyl-acetic acid. Kelsoe and Cerny (51) have demonstrated a reciprocal expansion of antigen activated idiotype bearing clones of lymphocytes followed by expansion of clones which bear anti-id receptors in Balb/c mice immunized to Streptococcus pneumonia. They hypothesized that the out of phase expansion of the reciprocal cell sets was the result of interactions of id and anti-id. The production of auto-anti-id in man has been demonstrated to occur during the immune response against tetanus toxoid. The presence of this anti-id was associated with the loss of some of the anti-tetanus toxoid idiotypes (52). Naturally occurring anti-id has also been demonstrated in myasthenia gravis patients using, as the idiotype probe, a mouse monoclonal antibody. Those patients with the highest titer of anti-receptor antibody had the lowest level of anti-id, while in patients with the lowest titer of anti-receptor antibody (id), the highest titer of anti-id was detected (53). Comparable findings have been reported in patients with anti-DNA antibody and reciprocal anti-id in systemic lupus

erythematosus (54) and in some IgA-deficient people in terms of anti-casein antibody and its reciprocal anti-id (55). In these later experiments the anti-id was detected using homologous antibody as the id probe.

These experiments suggest that because anti-id is present during a normal immune response and regulates the expression of ids, anti-id may be an important part of the regulation of the immune response.

In reference to IgE, Geczy and his associates (58) have shown that in guinea pigs, the administration of syngeneically derived antibody led to a marked suppression in the IgE level as measured by passive cutaneous anaphylaxis. This treatment also resulted in the production of anti-id and if this anti-id was given to a guinea pig followed by antigen stimulation, there was a marked suppression in the subsequent response. This group has shown that in the mouse, the preexisting anti-hapten IgE and IgG antibody response could be suppressed with either anti-hapten or anti-carrier anti-idiotypic antibody (59-61).

These experiments and others like them show that id/anti-id interaction results usually in suppression of the immune response. However, this is not always the case. For example, Eichmann and Rajewsky (62) showed that the injection of guinea pig IgG_1 anti-id would enhance the expression of id designated A5A when stimulated with

Streptococcus whereas if the anti-id was an IgG_2, the expression of A5A id was suppressed. Recently, Forni et al. (63) showed that the injection of anti-SRBC IgM into normal mice induced plaque forming cells of the same specificity as the injected antibody. Further analysis established that the mechanism of this enhanced responsiveness was based on id/anti-id interactions (63,64). The authors state that "these results support network concepts. Thus if an antigen specific response can be induced solely by using components of the immune system itself, it follows that, in its basic economy, this system is autonomous and does not depend on the introduction of antigen to adjust to new dynamic states" (63 p. 1127). In this case anti-id most probably acted as an internal image of antigen. There have been other examples that demonstrated the mimicry of antigen by antibody. For example, Sege and Peterson (65) showed that anti-id prepared against antibody to insulin could mimic the action of insulin in cells. Schreiber et al. (66) showed that anti-id against rabbit antibodies to alprenolol would compete with alprenolol for the binding site on turkey red blood cells. This anti-id could also stimulate adenylate cyclase activity in the cells.

This discussion raises the possibility that the administration of autologous antibody might regulate antigen specific IgE response in the dog through id/anti-id

networks. Therefore the objectives of the work presented here were

1) To develop a consistent IgE antibody response in the dog and to study the kinetics of this response.

2) To examine the effects that autologous antibody administration had on an ongoing IgE response.

3) To determine if an anti-id response occured at any point during the experiment and if so, to examine the relationship between ids and anti-ids.

CHAPTER TWO
THE INDUCTION AND KINETICS OF AN ANTI-DNP IGE RESPONSE

Introduction

The value of the dog as an experimental model to study atopy has been described. However, the expense and difficulty of obtaining atopic dogs necessitated the development of a system in which antigen-specific IgE could be consistently induced. The use of a hapten-coupled carrier as an antigen was felt to be more convenient than a more complex, heterogenous substance such as an allergen to study the synthesis and regulation of IgE antibody. Furthermore, Halliwell (7) and Schwartzman et al. (67) have shown that two dogs immunized with dinitrophenol coupled to ascaris antigen and administered in aluminum hydroxide as the adjuvant, developed anti-DNP IgE antibody. However, it is not known a) if all dogs so immunized produce IgE antibody, b) how long the detectable IgE response remains, and c) what the immune response in terms of other isotypes might be. The purpose of the following experiments, then, was to induce a consistent anti-hapten IgE antibody

response and to examine the kinetics of the IgE, IgG and IgM anti-hapten antibody response.

Materials and Methods

Protein Concentration Determination

The concentration of immunoglobulin was determined from known molar extinction coefficients and by its ability to absorb light at 280 nm. Alternatively, the protein concentration was determined at 595 nm using Bradford's reagent (68) and interpolated from a standard curve derived from the absorption values of a series of dilutions of a similar freeze dried purified protein of known concentration. The measurements with both techniques gave concordant results.

Antigens

Azobenzenarsonate coupled to keyhole limpet hemocyanin (ABA-KLH) was a gift from Dr. Mark Greene, Harvard University. Ascaris antigen was prepared from adult Toxocara canis by the method of Strejan and Campbell (69) and modified as follows: Fifty adult T. canis were obtained from the gastrointestinal tract of euthanized dogs. The worms were washed with phosphate buffered saline (PBS), pH 7.2, containing 0.02 percent sodium azide, ground with a mortar and pestle and incubated for 48 hours at 4° C. Large particulate matter was removed by centrifugation

at 1000 x g for ten minutes in an IEC centra-7R centrifuge (International Equipment Co.) The supernatant was then centrifuged at 49000 x g for one hour in an L8-70 ultra-centrifuge (Beckman Instrument Co., Norcross, Ga.) to remove fine particles and was then chromatographed through a Sephadex G-100 column (Pharmacia Fine Chemicals, Piscataway, N.J.). The first peak was pooled, concentrated by negative pressure dialysis, dialyzed against PBS, pH 7.2, passed through a filter having 0.2 micron pores filter (Acrodisc, Gelman Co., Ann Arbor, Mi.) and used as the ascaris antigen (ASC). Human serum albumin (HSA) fraction V was obtained from Sigma Chemical Co. (St. Louis, Mo.). Bovine gamma globulin (BGG) was prepared from serum of an adult cow by precipitation with 40 percent saturated ammonium sulfate. The precipitate was dialyzed against 0.035 M phosphate buffer, pH 8.0 and was then chromatographed through a diethylaminoethyl cellulose (DEAE) ion exchange column (DEA, DE52, Whatman Chemicals, Kent, England) equilibrated with this same buffer. The effluent protein was concentrated by negative pressure dialysis and dialyzed against PBS, pH 7.2.

Dinitrophenylation of Proteins

Dinitrophenylation of protein was performed by mixing equal weights of protein, potassium carbonate (Fisher Scientific Co., St. Louis, Mo.) and

2,4-dinitrobenzenesulphonic acid (DNP) (Eastman Kodak Co., Rochester, N.Y.) were mixed in distilled water (70). This was then incubated while gently stirring for 18 hours at room temperature. The solution was chromatographed through a Sephadex G-25 column (Pharmacia Fine Chemicals, Piscataway, N.J.) to separate bound from free DNP. The dinitrophenylated protein was concentrated by negative pressure dialysis and extensively dialyzed against PBS, pH 7.2. The extent of substitution was estimated by measuring light adsorption at 360 nm and assuming a molar extinction coefficient of 1.75×10^4 for the dinitrophenyl group. The average epitope density expressed as molecules of DNP per molecule carrier was DNP_{27}-HSA, $DNP_{14.8}$-BGG. Since ASC extract was a complex mixture of proteins, the extent of substitution was expressed as moles DNP/mg ASC and was 6.32×10^{-5} DNP/ASC. A single batch of each of these antigens was prepared and used throughout the experiment. These antigens, when not in use, were stored at -70^{o}C. The degree of substitution did not change due to storage.

Aluminum hydroxide Precipitation of Protein

Aluminum hydroxide precipitation of protein was performed by mixing one part of a 5 percent sterile solution of aluminum potassium sulfate ($AlK(SO_4)_2$), Mallincrodt, Paris, Kentucky) with five parts of 1 mg/ml

solution of protein (70). The pH was then adjusted with 0.1 N NaOH to pH 6.3 to ensure adequate precipitate.

Affinity Chromatography

Sepharose 4B beads (Pharmacia Fine Chemicals) were activated using cyanogen bromide (CnBr) by adding 1.5 grams CnBr in 20 ml distilled water to 10 ml of washed Sepharose 4B beads and adjusted to pH 11 with I N NaOH. This mixture was maintained on ice at pH 11 for 6 minutes after which the beads were washed with 100 volumes of iced cold water. Ninety milligrams of protein in 6 ml PBS, pH 7.2 were added and incubated for 12 hours at $4^{o}C$. Alternatively pre-activated Sepharose 4B beads were obtained (Pharmacia Fine Chemicals) and protein was bound to these beads as described by the manufacturer. To remove unbound protein in both cases, the beads were washed with five alternate cycles of 0.1 M Tris buffer, pH 8.3 containing 0.5 M NaCl followed by 0.1 M glycine HCl, pH 2.8. Any remaining sites were blocked by incubating the beads in 0.1 M Tris buffer, pH 8.3 for four hours at room temperature. The column was then flushed with normal canine serum and washed as described above.

Pepsin Digestion and Purification of $F(ab)'_2$ Antibody Fragments.

The usual procedure for $F(ab)'_2$ digestion of immunoglobulin was to digest the antibody with 6 percent pepsin (w/v) in 0.2 M acetate buffer, pH 4.5 for 18 hours at 37 ^{o}C. However, this process resulted in some loss of antigen binding of the $F(ab)'_2$ presumably from the prolonged incubation time at pH 4.5. Where maintenance of this activity was critical, protein was digested with 20 percent pepsin w/v in 0.2 M acetate buffer pH 4.5 for five hours at 37^{o}C. The digested protein was separated from Fc pieces and intact antibody by passage through a cyanogen bromide-activated heavy chain specific immunoabsorbent column followed by passage through a Staphylococcus protein A affinity column (Pharmacia Fine Chemicals). The effluent was concentrated by negative pressure dialysis and dialyzed against PBS, pH 7.2.

Antisera

a) Preparation and purification of anti-IgG. Normal canine serum (NCS) was precipitated with a 40 percent saturated solution of ammonium sulfate. The precipitate was dialyzed against 0.035 M phosphate buffer, pH 8.0 and applied to a DEAE ion exchange column equilibrated with this same buffer. The effluent protein was concentrated by

negative pressure dialysis. One milligram of this material was emulsified in complete Freund's adjuvant (CFA) and administered intramuscularly to rabbits at two week intervals four times. Fifty milliliters of blood were obtained from the rabbit by ear vein venapuncture every two weeks starting after the second immunization. All serum which gave visible precipitation reactions by agar-gel diffusion against canine IgG was pooled. This antisera was passed through a cyanogen bromide-activated sepharose 4B $F(ab)'_2$ affinity column, to remove light chain activity, followed by adsorption to and elution with alycine HCl (0sl M), pH 2.8 from a canine IgG bound affinity column. This anti-IgG detected three subclasses of canine IgG (IgG_1, IgG_{2ab}, IgG_{2c}) but no other protein as measured in an immunoelectrophoresis (70) of NCS (figure 1). To determine if this antiserum detected IgE, the antiserum was radiolabelled and used in a radio-immunoassay. The serum sample tested contained both anti-DNP IgG and anti-DNP IgE. Therefore, an aliquot of this serum was heat inactivated and the level of anti-DNP IgG was compared in this aliquot to a second aliquot of this serum that was not heat inactivated. Additionally, anti-canine IgE was added to an aliquot of this sample to determine if this unlabelled anti-IgE might compete with the anti-IgG for Fc binding sites. Heating serum for four hours at $56^{\circ}C$ destroys the heavy chain antigenic

determinants of canine IgE (7). The level of anti-DNP antibody increased both when the serum was inactivated and when non-labelled anti-IgE antiserum was added to the sample. This indicates that this anti-IgG antiserum has minimal, if any, anti-IgE activity.

b) Preparation and purification of anti-IgE. A 40 percent saturated ammonium sulfate precipitate of serum obtained, from a dog that was heavily parasitized and presumed to have high levels of IgE, was dialyzed against 0.035 M phosphate buffer pH 8.0 and applied to a DEAE cellulose column equilibrated with this same buffer. The effluent protein was concentrated by negative pressure dialysis and applied to a set of three in series Sephacryl S-200 columns (Pharmacia Fine Chemicals, Piscataway, N.J.). The first one-third of the second protein peak, which was the IgE-rich fraction as determined by agar-gel immunoprecipitation, was collected, concentrated by pressure dialysis and reapplied to these columns. The resulting IgE-rich fraction was collected and used to immunize rabbits as described previously. The rabbits were bled as described above. Serum that produced visible precipitation lines against the immunizing antigen in an agar gel immunodiffusion were pooled. The resulting antiserum detected both IgE and IgG by immunoelectrophoresis. It was rendered specific for the former protein by passage through an

Figure 1.

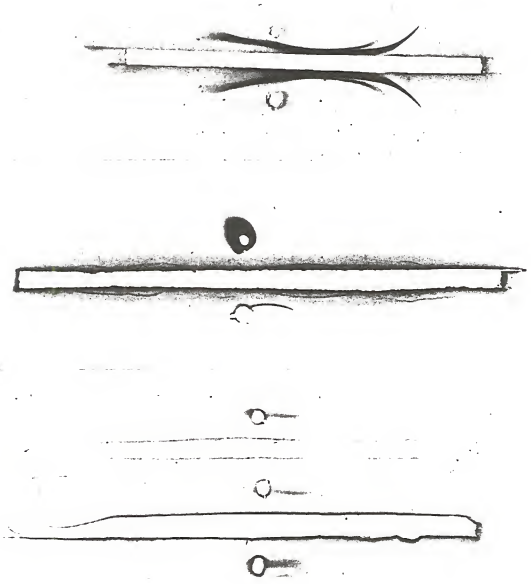
The specificity of anti-canine IgG as assayed in an immunoelectorphoresis against normal canine serum.

Figure 2.

The specificity of anti-canine IgE as assayed in an immunoelectrophoresis against normal canine serum (bottom well) and this same serum after heat inactivation (top well).

Figure 3.

The specificity of anti-canine IgM as assayed in an immunoelectrophoresis against normal canine serum. The anti-canine IgM in the bottom through is before adsorption with the supernatant of a 50 percent saturated ammonium sulfate precipitate of normal canine serum. The top trough has the anti-canine IgM antiserum after this treatment.



affinity column made with the heat inactivated immunogen which removed all antibody except anti-IgE antibody. Purified antibody was then prepared by adsorption to and elution from an IgE-rich affinity column. This purified antiserum detected a single heat-labile protein by immunoelectrophoresis (figure 2), produced reverse cutaneous anaphylaxis in dogs at a high dilution of serum (10^{-6}) and was unable to detect canine anti-DNP IgG in a RIA indicating that it had no specificity for this antibody.

c) Preparation and purification of anti-IgM. Canine IgM myeloma serum, which contained approximately 58 mg/ml IgM was chromatographed on Sephacryl S-200 and the void volume was collected to obtain IgM. Two milligrams of this material was emulsified in CFA and injected intramuscularly at four sites into sheep. This was repeated at two week intervals five times. Five hundred milliliters of blood were collected by jugular vein venapuncture every two weeks. Sera that produced precipitation lines against the immunizing antigen, in an agar-gel diffusion against the immunizing antigen, were pooled. Light chain activity was removed from the antiserum by passage through a canine IgG affinity column. Antibody was then purified by adsorption to and elution from an IgM affinity column. The eluted proteins produced two bands on immunoelectrophoresis of NCS, one of which was IgM and the other an unknown protein.

This second activity was removed by adsorption with the supernatant of a 50 percent saturated ammonium sulfate precipitation of NCS (figure 3). This antiserum was assayed for anti-DNP IgE and IgG activity by RIA. Serum that was used contained both of these antibody isotypes. No antibody was detected indicating the antiserum did not have activity for IgE or IgG.

Isotope Labelling of Protein

Two methods were used to label proteins with radioactive iodine. In the first method, between 1 and 2 mg of protein in 0.1 ml PBS pH 7.0 without azide and .5 mCi ^{125}I (Amersham, Chicago, Il.) was incubated on ice with 15 µl KI(0.1 mM) and 30 µl chloramine T (10 mM) for 15 minutes. After this incubation, 25 µl sodium metabisulphate (10 mM) and 50 µl KI(100 mM) were added to stop the reaction. To separate bound and free iodine, the material was chromatographed through a G-25 Sephadex column. The first peak containing radiolabel was pooled, concentrated and dialysed against PBS, pH 7.2. Alternatively, one Iodobead^{R} (Pierce Chemical Co., Rockford, Il.) was added to 100 µg of protein in PBS, pH 7.0 and 0.5 mCi ^{125}I. After a fifteen minute incubation, the bound and unbound ^{125}I was separated as described earlier. The specific activity of the radiolabelled antibody was usually about 300 µCi/mg protein (range 212-496).

Radioimmunoassay for the Detection of DNP Specific Antibody

Microtiter wells (Immulon Remov-a-well Strips^{R}, Dynateck, Richmond, Va.) were coated with 50 µl of 20 µg/ml dinitrophenylated bovine gamma globulin (DNP-BGG) in Tris buffer, pH 8 (0.1 M Tris, 0.15 M NaCl). After incubating for 12 hours at 4^{o}C the wells were then washed three times with this buffer. Any remaining sites were blocked with 2.0 percent HSA in PBS containing 0.5 percent Tween 20 for three hours at room temperature. Phosphate buffered saline, pH 7.2 containing 0.5 percent Tween 20 and 2.0 percent HSA is referred to as RAST+ and this same buffer without HSA is called RAST-. Serum samples were diluted in PBS, pH 7.2 added to the appropriate wells, incubated for three hours at 4^{o}C followed by five washes with RAST-. Approximately 50,000 counts per minute (cpm) of radiolabelled antiserum in RAST- was added to the well, incubated for three hours at 4^{o}C and washed three times with RAST-. The radioactivity associated with each well was determined in a Searle-Packard gamma counter (Chicago, Il.). Each sample was assayed in triplicate and each sample was counted for one minute. The maximum number of cpm bound was about 20% of the amount added. The background activity was determined by including in each assay the following controls: 1) A set of triplicate wells in which BGG rather than DNP-BGG was used as the antigen, 2) A

triplicate set of wells in which PBS rather than serum was added. The mean cpm from these controls were subtracted from the cpm of the test sample. Although the values varied from experiment to experiment, the maximum cpm of these controls were consistently lower than the lowest values obtained for test samples.

A standard serum sample was included with each assay as an internal reference. An arbitrary antibody concentration was determined by assigning a value of 64 units to the undiluted standard IgG and IgE sample and 32 units to the undilute IgM standard. By interpolating from the linear portion of the standard curve, the relative units of antibody for test samples were calculated.

Animals and Immunization Schedule

Outbred pregnant female dogs were obtained from the Division of Animal Resources, University of Florida. Serum from these dogs was screened by RIA to ensure that they did not have anti-DNP antibody at the time of whelping. The puppies of these bitches were used as experimental animals. Serum samples were obtained on the day of birth and weekly therafter. Each puppy received 100 µg aluminum hydroxide precipitated dinitrophenol coupled ascaris antigen by the intraperitoneal route on the day of birth and at two week intervals on three further occasions. Each

dog received a distemper-hepatitis modified live virus vaccination at week four and eight.

Results

Standard Curve

The relative antigen-specific antibody concentration was determined by interpolation from the linear portion of the standard curve included with each assay. This serum sample contained high levels of the isotype under investigation. An example of a standard curve for anti-DNP IgE, IgG and IgM is given in figures 4, 5 and 6.

Antibody Response

Twenty-eight dogs immunized with 100 μg DNP-ASC in aluminum hydroxide developed an IgE, IgG and IgM serum antibody response. The mean relative antibody concentration for the three isotypes is depicted in figure 7, 8 and 9. The IgM response usually was highest in samples taken seven days after the first injection of antigen. However, as seen in table 1, eight of the dogs (5,7,8,12,14,16,23,25) had anti-DNP IgM concentrations that were greatest in samples obtained at two weeks and three dogs (9,17,22) after three weeks. Four weeks after the first antigenic challenge, five dogs had no detectable IgM antibody and

Figure 4.

Dilutions of the standard anti-DNP IgE serum sample assayed by RIA. The bars represent the standard deviation of the mean.

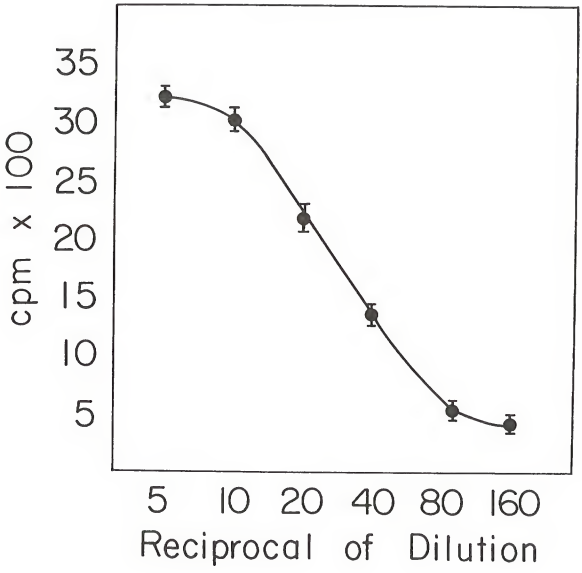

35
30
25
20
15
10
5
cpm x 100
5
10
20
40
80
160
Reciprocal of Dilution

Figure 5.

Dilutions of the standard anti-DNP IgG serum sample assayed by RIA. The bars represent the standard deviation of the mean.

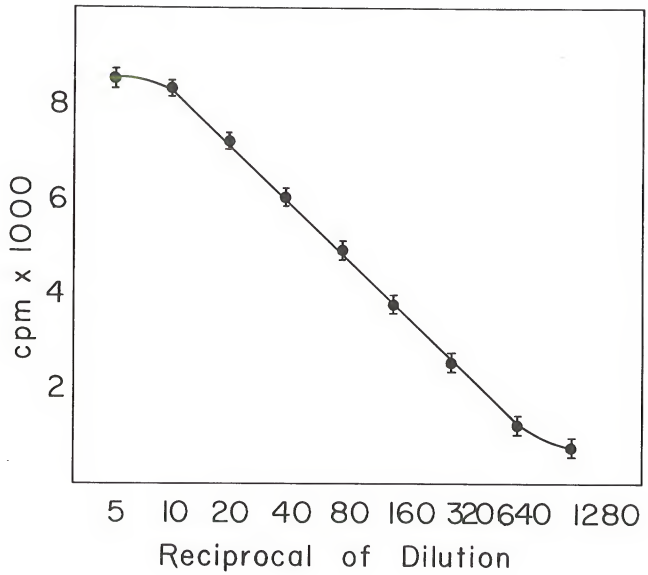
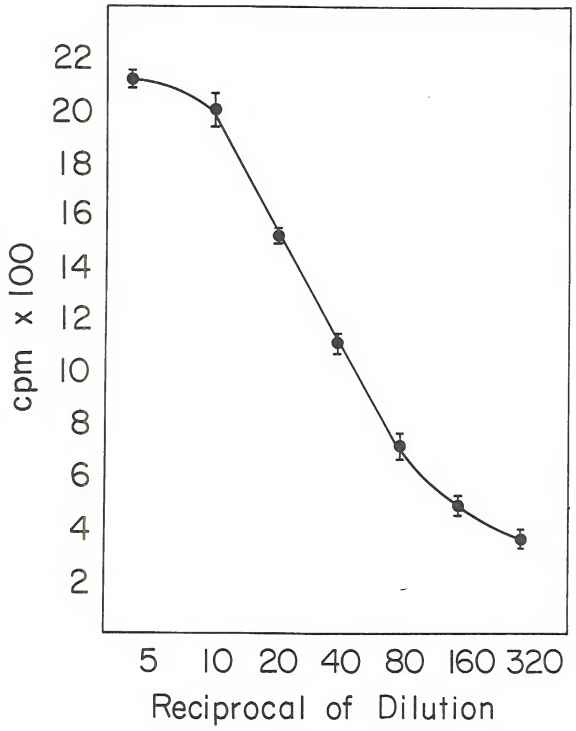

8
6
4
2
cpm x 1000
5
10
20
40
80
160
320
640
1280
Reciprocal of Dilution

Figure 6.

Dilutions of the standard anti-DNP IgM serum sample assayed by RIA. The bars represent the standard deviation of the mean.


22
20
18
16
14
12
10
8
6
4
2
cpm x 100
5
10
20
40
80
160
320
Reciprocal of Dilution

after six weeks the levels of IgM antibody fell to background despite maintenance of the immunizing protocol.

The anti-DNP IgE and IgG antibody responses followed similar kinetics to each other. There was an initial lag of two weeks before antibody of these classes was detected (figures 8 and 9). At the time of the second immunization (two weeks after the primary immunization), there was a sharp rise in the antibody levels which continued for one additional week. Thereafter, the antibody concentration was maintained at that level or started to gradually decline. As was the case in the IgM antibody response, some dogs deviated from the general trend. Two dogs had detectable IgE antibody levels one week after primary immunization (Table 2) whereas three dogs failed to develop a detectable response until after the third week and, in the case of one dog, IgE antibody was not detected until the fifth week from primary immunization. The IgE antibody response persisted through the seven week course of the experiment in all dogs. Anti-DNP IgG antibody was detected in nine dogs one week after primary immunization (table 3) and by the fourth week, all dogs had an IgG antibody response. Detectable IgG persisted throughout the immunization schedule but there was a gradual decline in IgG antibody levels towards the end of the immunizing schedule (See figure 9, table 3).

Figure 7.

The mean relative anti-DNP IgM concentration in 28 dogs as measured by RIA. Each dog was immunized biweekly four times starting at week 0 with DNP-ASC (see text for further details). The antibody concentration was calculated from an arbitrary antibody concentration scale derived from the titration of a standard anti-DNP IgM containing serum.

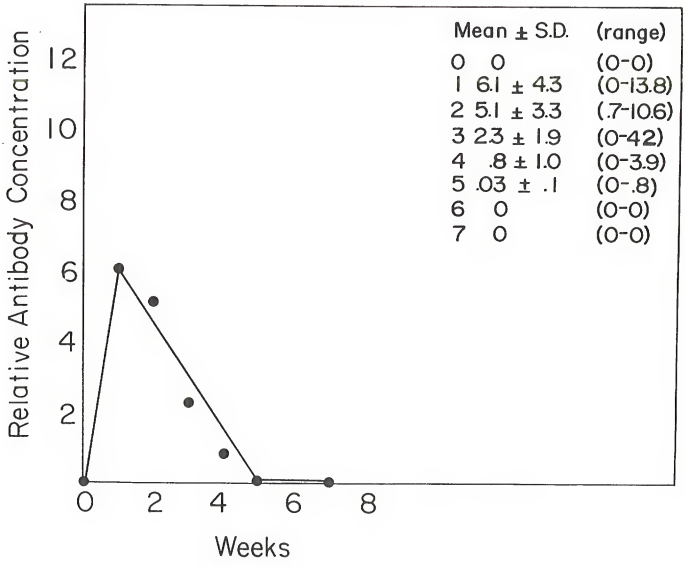

Relative Antibody Concentration
12
10
8
6
4
2
0
2
4
6
8
Weeks
Mean ± S.D. (range)
0 0 (0-0)
1 6.1 ± 4.3 (0-13.8)
2 5.1 ± 3.3 (.7-10.6)
3 2.3 ± 1.9 (0-4.2)
4 .8 ± 1.0 (0-3.9)
5 .03 ± .1 (0-.8)
6 0 (0-0)
7 0 (0-0)

Figure 8.

The mean relative anti-DNP IgE concentration in 28 dogs as measured by RIA. Each dog was immunized biweekly four times starting at week 0 with DNP-ASC. The antibody concentration was calculated from an arbitrary antibody concentration scale derived from the titration of a standard anti-DNP IgE containing serum. (See text for further details.)

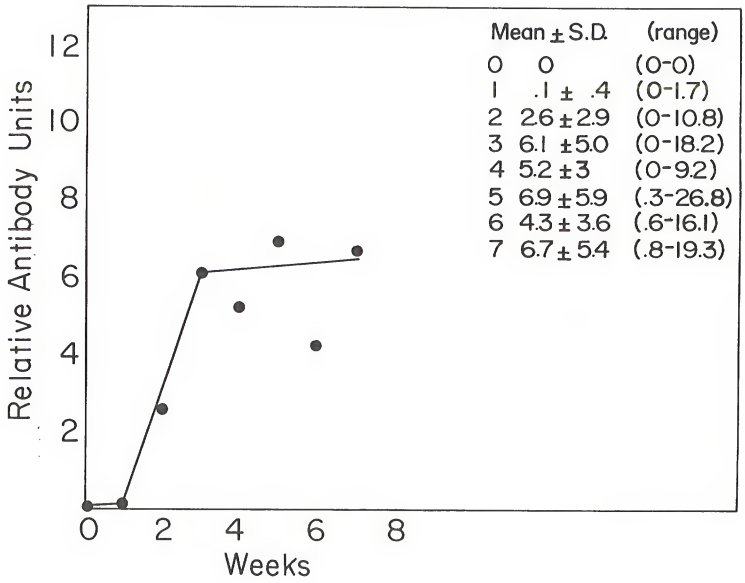

Relative Antibody Units
12
10
8
6
4
2
0
2
4
6
8
Weeks
Mean ± S.D. (range)
0 0 (0-0)
1 .1 ± .4 (0-1.7)
2 2.6 ± 2.9 (0-10.8)
3 6.1 ± 5.0 (0-18.2)
4 5.2 ± 3 (0-9.2)
5 6.9 ± 5.9 (.3-26.8)
6 4.3 ± 3.6 (.6-16.1)
7 6.7 ± 5.4 (.8-19.3)

Figure 9.

The mean relative anti-DNP IgG concentration in 28 dogs as measured by RIA. Each dog was immunized biweekly four times starting at week 0 with DNP-ASC. The antibody concentration was calculated from an arbitrary antibody concentration scale derived from the titration of a standard anti-DNP IgG containing serum (see text for further details).

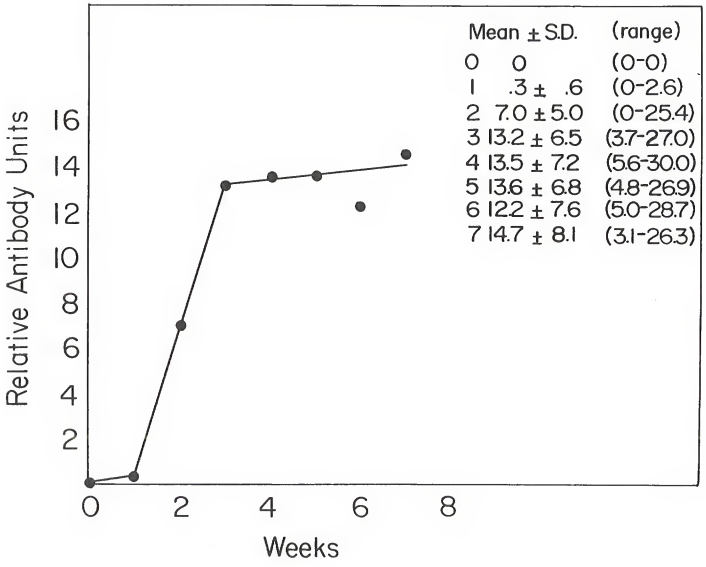

Relative Antibody Units
16
14
12
10
8
6
4
2
0
2
4
6
8
Weeks
Mean ± S.D. (range)
0 0 (0-0)
1 .3 ± .6 (0-2.6)
2 7.0 ± 5.0 (0-25.4)
3 13.2 ± 6.5 (3.7-27.0)
4 13.5 ± 7.2 (5.6-30.0)
5 13.6 ± 6.8 (4.8-26.9)
6 12.2 ± 7.6 (5.0-28.7)
7 14.7 ± 8.1 (3.1-26.3)

Table 1
The Relative Anti-DNP IgM Concentration in 28 Dogs

Animal Number / Weeks	1	2	3	4
0	0	0	0	0
1	16.6±.87	4.4±.39	6.5±.47	4.5±.06
2	10.6±1.23	3.2±.23	3.4±.24	4.4±.27
3	.3±0	1.4±.56	0	.6±.14
4	.2±.01	.2±0	0	.1±.03
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
Animal Number / Weeks	**5**	**6**	**7**	**8**
0	0	0	0	0
1	2.9±.25	6.4±.82	6.8±1.01	3.1±.28
2	5.8±.90	5.6±.39	7.8±.32	5.2±.17
3	0	5.1±.07	3.4±.31	0
4	0	3.9±.24	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
Animal Number / Weeks	**9**	**10**	**11**	**12**
0	0	0	0	0
1	0	10.9±1.03	7.0±.02	5.0±.25
2	2.0±.14	3.0±.41	2.8±.46	5.1±.37
3	3.3±.29	.8±.01	1.8±.19	2.0±.21
4	0	0	1.0±.07	1.8±.27
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
Animal Number / Weeks	**13**	**14**	**15**	**16**
0	0	0	0	0
1	5.5±.16	1.8±.18	10.1±.79	1.3±.12
2	5.0±.23	5.2±.33	6.1±.43	2.6±.37
3	4.8±.07	2.3±.16	1.8±109	2.5±.14
4	.9±.13	.5±.22	1.6±.17	1.2±.20
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0

Table 1 Continued

Animal Number / Weeks	17	18	19	20
0	0	0	0	0
1	1.1±.03	14.8±2.01	3.9±.39	2.7±.22
2	4.4±.17	5.6±.81	2.1±.09	2.1±.40
3	6.0±.31	3.1±.23	.8±.02	0
4	1.7±.15	2.1±.10	0	0
5	.8±.0	0	0	0
6	0	0	0	0
7	0	0	0	0
Animal Number / Weeks	**21**	**22**	**23**	**24**
0	0	0	0	0
1	8.7±.83	.7±.15	8.1±.44	5.1±.46
2	7.9±.09	4.5±.18	12.6±.99	.6±.06
3	3.2±.48	6.2±.46	2.7±.23	0
4	1.6±.06	2.1±.05	0	0
5	.1±0	0	0	0
6	0	0	0	0
7	0	0	0	0
Animal Number / Weeks	**25**	**26**	**27**	**28**
0	0	0	0	0
1	4.0±.09	13.8±.1.35	5.2±.29	10.6±.87
2	6.2±.21	9.1±1.37	.7±0	x
3	2.5±.30	3.4±.40	1.0±.16	4.2±.36
4	0	2.1±.11	0	.8±.023
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0

a) Each dog was immunized with DNP-ASC in adjuvant at weeks 0,2,4 and 6.

b) The units were calculated from a relative antibody concentration scale derived from the titration of a serum sample containing anti-DNP IgM. A value of zero indicates no detectable anti-DNP IgG. The data was the mean antibody concentration of a sample run in triplicate ± the standard deviation from the mean. This was calculated by adding and subtracting the standard deviation to the mean cpm and calculating the relative antibody concentration for these numbers. These numbers were then subtracted from the mean concentration.

Table 2
The Relative Anti-DNP IgE Concentration In 28 Dogs
Following Immunization with DNP-ASC a)

Animal Number / Weeks	1	2	3	4
0	0 b)	0	0	0
1	0	0	0	0
2	1.2±.31	10.8±1.90	3.2±.36	3.2±.21
3	1.5±.27	11.3±.27	10.0±.76	4.8±.07
4	1.7±.16	9.2±.36	8.5±.44	4.6±.29
5	1.3±.49	11.6±.24	10.1±.83	5.1±.36
6	1.0±.22	5.6±.70	3.3±.12	3.3±.16
7	3.0±.96	7.7±1.01	7.0±.41	4.9±.37
Animal Number / Weeks	**5**	**6**	**7**	**8**
0	0	0	0	0
1	.6±.03	0	0	0
2	3.6±.46	1.9±.17	3.2±.10	.6±.07
3	11.9±.99	13.4±1.35	11.8±.77	5.0±1.00
4	7.8±1.04	8.0±.61	6.7±.83	3.9±.43
5	9.0±.63	12.7±.62	11.8±2.45	15.2±.18
6	11.0±.71	16.1±.90	5.5±.69	7.6±.45
7	14.1±1.42	12.8±.88	2.8±.25	6.6±.51
Animal Number / Weeks	**9**	**10**	**11**	**12**
0	0	0	0	0
1	1.7±.39	0	0	0
2	5.6±.26	0	2.1±.24	7.5±.88
3	18.2±2.73	3.1±.69	8.3±.74	13.2±1.86
4	8.4±.97	5.5±.43	7.8±.79	9.1±1.02
5	10.0±.64	5.2±.47	3.9±.81	26.8±1.30
6	7.1±.31	3.0±.30	2.4±.65	7.0±1.51
7	8.7±.51	12.6±.76	4.8±2.38	15.4±.36
Animal Number / Weeks	**13**	**14**	**15**	**16**
0	0	0	0	0
1	0	0	0	0
2	0	1.8±.36	1.0±.11	0
3	0	9.5±.37	3.4±.48	.7±.12
4	0	8.4±.67	7.5±1.06	3.9±.61
5	.3±.11	12.0±.87	5.1±.56	3.9±.14
6	.8±.23	8.5±.21	.6±.01	3.7±.37
7	1.6±.52	19.3±1.98	5.2±.50	4.5±.66

Table 2 Continued

Animal Number / Weeks	17	18	19	20
0	0	0	0	0
1	0	0	0	0
2	.2±.07	10.1±.21	0	3.3±.09
3	11.0±.96	9.3±.49	1.4±.08	4.2±.15
4	7.7±.14	8.9±.47	.3±.04	4.8±.33
5	9.8±.34	9.4±.67	1.0±.20	5.2±.40
6	2.2±.26	6.9±.52	1.5±.08	5.8±.26
7	6.7±.31	15.3±.89	2.3±.21	7.1±.43
Animal Number / Weeks	**21**	**22**	**23**	**24**
0	0	0	0	0
1	0	0	0	0
2	3.0±.10	1.5±.17	2.1±.13	.1±.04
3	4.5±.11	2.6±.12	1.7±.09	1.0±.06
4	4.0±.27	3.0±.31	1.8±.14	1.0±.17
5	5.6±.36	2.6±.21	1.0±.13	1.6±.31
6	2.3±.12	2.8±.32	0.8±.07	0.9±.07
7	4.8±.22	2.6±.60	0.9±.21	1.3±.29
Animal Number / Weeks	**25**	**26**	**27**	**28**
0	0	0	0	0
1	0	0	0	0
2	1.0±.29	1.7±.37	.6±.05	x
3	2.7±.31	2.1±.05	3.2±.22	1.1±.06
4	2.0±.26	3.0±.42	2.0±.23	7.3±.26
5	1.8±.19	1.3±.12	1.0±.15	8.8±.51
6	.7±.09	4.3±.27	1.5±.13	3.3±.16
7	1.2±.10	1.7±.28	0.8±.06	8.4±.42

a) Each dog was immunized with DNP-ASC in adjuvant at weeks 0,2,4 and 6.
b) The units were calculated from relative antibody concentration scale derived from the titration of a serum sample containing anti-DNP IgE. A value of zero indicates no detectable anti-DNP IgE. The data was the mean antibody concentration of a sample run in triplicate ± the standard deviation from the mean. This was calculated by adding and subtracting the standard deviation to the mean and calculating the relative antibody concentration for this number. The relative concentration for this number was subtracted from the mean concentration.

As would be expected for outbred animals, there was considerable variation in the immune response between dogs. If however, the IgG antibody concentration of dogs within single litters are examined, a more homogeneous trend is observed (table 4). There was, however, considerable animal-to-animal variation within a litter in the level of antigen specific IgE and IgM (tables 5 and 6). If these two litters are compared statistically, at each time point, using a student T test, there is a significant difference in the mean antibody level between the two groups in the IgG antibody after the first week (P is less than 0.001 in all instances).

When all the animals are considered, it appears that some are generally high responders to the antigenic stimulation whereas the response in other dogs is low. The high response or low response is seen for both IgG and IgE antibody classes in a single animal. For example, dogs 2 and 6 have a strong IgE and IgG response whereas dogs 15 and 24 have very weak responses. Although this trend predominates, this association of high responses or low responses is not always consistent, and a regression analysis comparing the level of anti-DNP IgG to the anti-DNP IgE failed to show a statistically significant correlation (p greater than 0.05).

Table 3
The Relative anti-DNP IgG Concentration in 28 Dogs
Following Immunization with DNP-ASC a)

Animal Number / Weeks	1	2	3	4
1	0 b)	0	0	0
2	0	0	.8±.02	0
3	4.1±.21	9.1±1.4	2.4±.21	3.7±.11
4	3.7±.20	15.5±.20	8.0±.10	7.4±.66
5	6.0±.64	16.4±1.26	4.8±.26	7.4±.14
6	8.1±.33	26.0±1.33	8.6±.23	4.8±.26
7	7.9±1.05	23.3±.14	6.7±.16	6.1±.88
Animal Number / Weeks	**5**	**6**	**7**	**8**
1	0	0	0	0
2	0.3±.02	0	0	0.1±0
3	3.7±.12	9.1±1.21	12.2±.77	25.4±1.31
4	8.6±.17	15.3±.86	11.1±.39	27.0±1.92
5	9.1±.34	17.0±1.41	9.2±.64	30.0±3.21
6	7.4±.23	25.6±.73	9.3±1.07	20.1±.49
7	8.6±.61	24.1±1.72	5.9±.26	8.7±1.26
Animal Number / Weeks	**9**	**10**	**11**	**12**
1	0	0	0	0
2	0	0	0	0
3	7.3±.69	4.3±.05	8.3±.80	6.3±.29
4	15.4±1.71	4.3±.12	12.4±.64	22.5±1.10
5	18.5±.86	5.6±.09	7.9±.32	20.8±.93
6	16.0±.46	7.1±.51	7.5±.93	17.8±1.79
7	8.3±.62	6.8±.19	5.5±.68	10.4±.23
Animal Number / Weeks	**13**	**14**	**15**	**16**
1	0	0	0	0
2	0	0	0	.3±.02
3	0	5.3±.31	3.8±.43	6.0±.11
4	10.1±.61	16.7±1.1	8.8±.16	4.4±.70
5	9.3±.75	14.3±.42	9.9±.63	11.6±1.60
6	7.8±.26	14.7±.32	8.9±.91	11.1±1.13
7	5.0±.16	10.4±.6	9.7±.46	17.2±.32

Table 3 Continued

Animal Number / Weeks	17	18	19	20
1	0	0	0	0
2	2.6±.13	.9±.04	.3±0	0
3	1.7±.14	1.7±.13	4.8±.17	11.7±.11
4	4.8±.36	22.7±.81	16.9±.65	12.8±.96
5	6.2±.58	24.9±1.65	21.3±.17	8.9±.04
6	6.9±.31	21.8±.77	20.8±1.16	7.6±.47
7	5.3±.40	28.2±1.24	21.9±.84	9.8±.36
Animal Number / Weeks	**21**	**22**	**23**	**24**
1	0	0	0	0
2	0	7.1±.33	12.9±.92	3.6±.24
3	5.6±.46	7.1±.33	12.9±.92	3.6±.24
4	5.4±.27	10.7±.75	18.0±.75	8.8±.43
5	8.5±.12	13.8±.99	13.9±.51	9.7±.81
6	10.6±.93	19.1±1.53	13.5±.87	9.0±.29
7	7.6±.46	18.2±.75	12.7±.60	6.6±.64
Animal Number / Weeks	**25**	**26**	**27**	**28**
1	0	0	0	0
2	0	0	.8±.07	.4±0
3	10.9±.47	3.3±.33	9.6±.48	x
4	10.0±1.79	16.3±.84	10.3±.68	12.9±.85
5	8.2±.97	15.4±.93	12.1±.06	22.8±1.68
6	7.8±.11	14.7±.43	18.7±1.78	26.9±1.41
7	5.0±.36	10.5±.70	19.6±.96	28.7±1.92

a) Each dog received immunization with DNP-ASC in adjuvant at weeks 0,2,4 and 6.

b) The units were calculated from a relative antibody concentration scale derived from the titration of a serum sample containing anti-DNP IgG. A value of zero indicates no detectable anti-DNP IgG. The data was the mean antibody concentration of a sample run in triplicate ± the standard deviation from the mean. This was calculated by adding and subtracting the standard deviation to the mean cpm and calculating the relative antibody concentration for these numbers. This number was then subtracted from the mean concentration.

Table 4
The Relative Anti-DNP IgG Concentration
In Two Litters of Dogs
Following Immunization with DNP-ASC a)

Litter 1

Weeks	3	4	17	20	24	25	M ± S.D b)
0	0	0	0	0	0	0	0
1	.8	0	2.6	0	0	0	.6 ± 1
2	2.4	3.7	1.7	11.7	3.6	10.9	5.7 ± 4.3
3	8.0	7.4	4.8	12.8	8.8	10.0	8.6 ± 2.7
4	4.8	7.4	6.2	8.9	9.7	8.2	7.5 ± 1.8
5	8.6	4.8	6.9	7.6	9.0	7.8	7.5 ± 1.5
6	6.7	6.1	5.3	9.8	6.6	5.0	6.6 ± 1.7
7	5.4	6.0	3.1	11.9	7.2	6.4	6.7 ± 2.9

Litter 2

Weeks	2	6	14	19	23	28	M + S.D.
0	0	0	0	0	0	0	0
1	0	0	0	.3	0	.1	0
2	9.1	9.1	5.3	4.8	12.9	x	8.2 ± 3.3
3	15.5	15.3	16.7	16.9	18.0	12.9	15.5± 1.8
4	16.4	17.0	14.3	21.3	13.9	22.8	17.6± 3.7
5	26.0	25.6	14.7	20.8	13.5	26.9	21.3± 5.9
6	23.3	24.1	10.4	21.9	12.7	28.7	20.2± 7.1
7	26.8	25.8	18.9	24.8	23.0	24.7	24.0± 2.8

a) The relative antibody concentration was determined by extrapolation of a standard serum sample. A value of zero indicates no detectable antibody activity. Each dog received DNP/ASC in adjuvant at weeks 0,2,4 and 6.
b) Mean ± standard deviation

Table 5
The Relative Anti-DNP IgE Concentration
In 2 Litters of Dogs
Following Immunization with DNP-ASC a)

Litter 1

Weeks	3	4	17	20	24	25	M + S.D. b)
0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
2	3.7	3.2	.2	3.3	.1	1.0	1.9 ± 1.7
3	10.0	4.8	11.0	4.2	1.0	2.7	5.6 ± 4.0
4	8.5	4.6	7.7	4.8	1.0	2.0	4.8 ± 3.0
5	10.1	5.1	9.8	5.2	1.6	1.8	5.6 ± 3.7
6	3.3	3.3	2.2	5.8	.9	.7	2.7 ± 1.9
7	7.0	4.9	6.7	7.1	1.3	1.2	4.7 ± 2.8

Litter 2

Weeks	2	6	14	19	23	28	M + S.D.
0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0
2	10.8	1.9	1.8	0	2.1	x	3.3 ± 4.3
3	11.3	13.4	9.5	1.4	1.7	1.1	6.5 ± 5.6
4	9.2	8.0	8.4	.3	1.8	7.3	5.8 ± 3.8
5	11.6	12.7	12.0	1.0	1.0	8.8	7.9 ± 5.6
6	5.6	16.1	8.5	1.5	.8	3.3	6.0 ± 5.7
7	7.7	17.8	19.3	2.3	.9	8.4	9.4 ± 7.7

a) The relative antibody concentration was determined by extrapolation of a standard serum sample. A value of zero indicates no detectable antibody activity. Each dog received DNP-ASC in adjuvant at week 0,2,4 and 6.
b) Mean ± Standard Deviation

Table 6
The Relative Anti-DNP IgM Concentration
in 2 Litters of Dogs
Following Immunization with DNP-ASC a)

Litter 1							
	3	4	17	20	24	25	M ± S.D. b)
Weeks							
0	0	0	0	0	0	0	0
1	6.5	4.5	1.1	2.7	5.1	4.0	4.0 ± 1.9
2	3.4	4.4	4.4	2.1	.6	6.2	3.5 ± 2.0
3	0	.6	6.0	0	0	2.5	1.5 ± 2.4
4	0	.1	1.7	0	0	0	.3 ± .7
5	0	0	.8	0	0	0	.1 ± .3
6	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0

Litter 2							
	2	6	14	19	23	28	M ± S.D.
Weeks							
0	0	0	0	0	0	0	0
1	4.4	6.4	1.8	3.9	8.1	10.6	5.9 ± 3.2
2	3.2	5.6	5.2	2.1	12.6	x	5.7 ± 4.1
3	1.4	5.1	2.3	.8	2.7	4.2	2.8 ± 1.6
4	.2	3.9	.5	0	0	.8	.9 ± 1.5
5	0	0	0	0	0	.1	.01 ± .04
6	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0

a) The relative antibody concentration was determined by extrapolation of a standard serum sample. A value of zero indicates no detectable antibody activity. Each dog received immunization with DNP-ASC in adjuvant at weeks 0,2,4 and 6.
b) Mean ± Standard Deviation

Discussion

The purpose of the experiments in this chapter was to induce an anti-DNP antibody response which included IgE and to examine the kinetics of this response. As described in the results section of this chapter, the anti-DNP IgE, IgG and IgM antibody response followed expected kinetics (71-73). The IgM response was present before IgE or IgG antibody was detected and disappeared after the sixth week in spite of continued antigenic challenge. The IgE and IgG production had a two week lag period in general, but once they developed, they were maintained throughout the immunization course.

If inbred laboratory animals such as mice are immunized with an antigen, a homogeneous response ordinarily results (74). However, in species that are genetically heterogeneous, such as man and dogs, the immune response to the antigen would be expected to be more highly variable (74). In these outbred dogs there was marked variation in the kinetics and magnitude of the antibody responses. The marked variations seen in these dogs are most probably the result of the genetic differences between them. In this context, it was noteworthy that the IgG response was more homogeneous within the same litter than between litters.

The genetic makeup of the animal also influences the class of antibody produced following antigen challange. In certain inbred animal strains, it is very difficult to mount an IgE antibody response without some type of manipulative process to eliminate T-suppressor cells (75,76). Furthermore, if a comparison is made between allergic and non-allergic people, a marked difference in antigen-specific IgE responsiveness to certain antigens is seen. Those individuals with allergic tendencies will have an enhanced IgE response to allergens whereas non-allergic people may not develop IgE antibody (77). There were notable differences between individual dogs in terms of their IgE response and in contrast to the IgG response there was no consistent pattern within litters. It is not clear if this failure to see similar patterns within a litter in the IgE level reflects the antigen chosen to study IgE in these dogs or if there are multiple genes that govern IgE levels in dogs. By having such a small sample size, a consistent pattern might not be observed for IgE levels. One of the dogs failed initially to develop an IgE titer. The IgE antibody response started after this dog was vaccinated with a modified-live canine distemper/hepatitis vaccine at four weeks of age. The immunization of dogs with this vaccine has been shown to enhance antigen-specific IgE response to an antigen administered at the same time (78). It has been hypothesized that this effect

is the result of a suppression of T-suppressor cells. Because the mechanism that would normally suppress IgE synthesis is altered, IgE antibody response will develop. This alteration in the suppressive network and subsequent IgE antibody synthesis has been called the "allergic breakthrough" (79).

Summary

Twenty-eight dogs immunized to DNP-ASC at birth and then three times at two week intervals produced serum anti-DNP antibody. The IgM response was detected one week after primary immunization and lasted for up to five weeks. The IgE and IgG antibody response in general was not present until week three but persisted through the immunization schedule. Although variation in the level and duration of the antibody response was detected between individual dogs, each dog did have a response that included all three isotypes examined.

Conclusions

(1) Dogs immunized with DNP-ASC develop a high level, long term IgE and IgG antibody response but the IgM response followed a different kinetic pattern in that it did not persist after week five.

(2) There was a difference in the responsiveness to this antigen seen between individual dogs for all antibody isotypes. This was most probably a reflection of the genetic heterogeneity between these dogs.

CHAPTER THREE
ATTEMPTS TO REGULATE AN ANTIBODY RESPONSE WITH AUTOLOGOUS ANTIBODY

Introduction

The mechanisms by which antibody responses are regulated have been studied extensively. Many experiments have shown that antibody can be self-regulating (80,81). There are at least two different ways that this can occur: 1) If antibody is present at the time of immunization, antibody can bind to and remove antigen. Therefore, the result would be a decrease or a failure to mount the response. 2) Antibody can induce an anti-idiotypic immune response which would regulate the subsequent expression of the antibody through id/anti-id interactions (80-82).

If the synthesis of IgE antibody could be suppressed with antibody, such therapy may be very beneficial in controlling IgE mediated allergic disease. As discussed in Chapter one, passively administered antibody in mice and rabbits has been shown to suppress IgE antibody (26,27). The purpose of the experiments in this chapter is to determine if the administration of autologous antibody has any effect on the ongoing antibody response in dogs.

Materials and Methods

Affinity Chromatography

Anti-DNP antibody was produced by immunizing dogs to DNP-ASC and was purified from serum by chromatography through a DNP-HSA affinity column as described in Chapter two. The bound antibody was eluted with 0.1 M glycine HCl, pH 2.5.

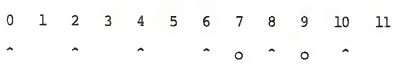
RIA

The RIA for detection of anti-DNP antibody was described in Chapter two.

Animals and Immunization Schedule

The same dogs that were described in Chapter two were used in these experiments. These dogs had received 100 µg of aluminum hydroxide precipitated DNP-ASC by the intraperitoneal route on the day of birth at two week intervals on three further occasions. Fifteen milliliters of serum were obtained from each dog at the time of final antigenic challenge. Anti-DNP antibody was purified from this serum by DNP-HSA affinity chromatography, concentrated to about 3 mg/ml by negative pressure dialysis and rendered bacterially sterile by passing through a filter having 0.2 micron sized pores. Dogs received either 10 or 100 µg of their own antibody emulsified in 2 ml of either complete

(CFA) or incomplete Freund's adjuvant (IFA). Dogs designated as controls received 2 ml of either CFA or IFA. In all cases, the injections were given at four sites subcutaneously seven and nine weeks after the first immunization with DNP/ASC. Animals were given DNP/ASC booster injections eight and ten weeks after the primary immunization (Fig.10).



^ = Administration of antigen

o = Administration of adjuvant with or without autologous antibody

Figure 10
Time Schedule for Immunizations

Results

DNP Affinity Column

There was no detectable anti-DNP antibody in the serum of any dog after passage through the DNP affinity column. On the other hand, the glycine HCl eluate contained high levels of anti-DNP IgG but no detectable anti-DNP IgM or IgE. Because anti-DNP IgE was not detected in either the effluent or the eluent from the affinity column but was detectable in the serum prior to such treatment, an aliquot of serum containing anti-DNP IgE was dialyzed against glycine HCl, pH 2.5 followed by dialysis against PBS, pH 7.2 to determine what effects glycine HCl had on canine IgE. There was no detectable anti-DNP IgE in this serum after such treatment as assayed by RIA.

Immunization with Autologous Antibody

As noted in Chapter two, there was considerable variation in the anti-DNP antibody respone between dogs. Tables 7 and 8 show the mean relative concentration of anti-DNP IgG and IgE respectively in each group of dogs prior to the autologous antibody administration and thereafter. These data are presented graphically in figures 11 and 12. The individual relative antibody concentrations

Table 7
The Mean Relative Anti-DNP IgG Concentration

Weeks	Group 1 a) N = 8	Group 2 N = 4	Group 3 N = 8
0	0	0	0
1	0	0	0
2	.2 ± .3	.5± .9	6.6 ± 1.7
3	8 .7 ± 7.6	4.4 ± 3.6	13.7 ± 7.5
4	12.1 ± 7.2	14.3 ± 6.3	13.2 ± 7.6
5	12.5 ± 8.4	13.3 ± 6.5	12.2 ± 5.6
6	13.7 ± 8.7	12.5 ± 6.0	7.8 ± 2.1
7	11.4 ± 7.7	13.4 ± 8.3	10.6 ± 5.9
8	12.6 ± 8.5	14.2 ± 8.6	12.1 ± 4.3
9	13.2 ± 7.9	14.5 ± 7.6	13.8 ± 5.9
10	13.2 ± 7.9	14.5 ± 7.6	13.8 ± 5.9
11	15.2 ± 9.2	17.8 ± 6.9	15.1 ± 7.4

Weeks	Group 4 N = 2	Group 5 N = 6	Control N = 8
0	0	0	0
1	0	.2 ± .3	.1 ± .3
2	6.4 ± 1.1	8.1 ± 4.4	7.6 ± 3.7
3	8.2 ± 3.5	12.7 ± 3.7	11.5 ± 4.1
4	11.2 ± 3.7	13.7 ± 5.2	13.1 ± 4.8
5	14.9 ± 6.0	15.1 ± 7.0	15.1 ± 6.4
6	12.9 ± 7.5	13.4 ± 8.9	13.6 ± 8.1
7	20.9 ± 6.4	16.4 ± 7.8	17.5 ± 7.3
8	20.1 ± 4.9	17.3 ± 8.5	17.8 ± 7.5
9	22.7 ± 8.5	18.9 ± 7.5	19.8 ± 7.3
10	23.5 ± 9.1	19.4 ± 8.0	20.5 ± 8.2
11	23.8 ± 10.7	17.7 ± 6.4	19.1 ± 7.0

a) All dogs received DNP/ASC immunization at 0,2,4,6,8 & 10 weeks. At 7 and 9 weeks: Group 1 received 10 µg autologous anti-DNP antibody in CFA, Group 2 received 100 µg autologous anti-DNP antibody in IFA, Group 3 received 100 µg autologous anti-DNP antibody in CFA. Group 4 received IFA alone, Group 5 received CFA only. Control values were the mean of groups 4 and 5.

Table 8
The Mean Relative Anti-IgE Antibody Concentration

Weeks	Group 1 a)	Group 2	Group 3
0	0	0	0
1	. 1 ± .2	0	.4 ± .85
2	3.5 ± 3.2	2.1 ± 3.5	3.8 ± 3.4
3	8.7 ± 4.3	4.9 ± 4.4	10.7 ± 6.5
4	6.3 ± 2.6	5.2 ± 3.6	7.7 ± 1.6
5	9.6 ± 4.5	5.8 ± 4.2	11.4 ± 10.5
6	6.7 ± 4.9	3.8 ± 3.0	3.7 ± 4.5
7	8.0 ± 5.3	7.8 ± 6.3	10.4 ± 4.6
8	7.2 ± 3.9	3.7 ± 2.7	7.8 ± 2.5
9	8.3 ± 3.2	5.3 ± 3.1	8.8 ± 1.0
10	7.6 ± 3.3	4.9 ± 2.6	7.3 ± 2.4
11	8.3 ± 3.8	5.4 ± 3.1	7.0 ± 2.7

Weeks	Group 4	Group 5	Control
0	0	0	0
1	0	0	0
2	2.3 ± 1.1	1.1 ± .8	1.4 ± 1.0
3	3.6 ± 1.3	2.0 ± .9	2.4 ± 1.2
4	3.5 ± .7	2.9 ± 2.3	3.0 ± 2.0
5	4.1 ± 2.1	2.6 ± 3.1	3.0 ± 2.8
6	2.6 ± .4	1.9 ± 1.5	2.1 ± 1.3
7	3.7 ± 1.6	2.4 ± 3.0	2.7 ± 2.6
8	6.6 ± 2.0	2.8 ± 1.9	3.8 ± 2.5
9	8.3 ± 1.0	3.2 ± 2.2	4.4 ± 3.0
10	7.1 ± .1	2.7 ± 2.2	3.8 ± 2.7
11	6.9 ± 2.3	3.0 ± 2.3	4.0 ± 2.7

a) All dogs received DNP/ASC immunization at 0,2,4,6,8 and 10 weeks. At 7 and 9 weeks: Group 1 received 10 μg autologous anti-DNP antibody in CFA, Group 2 received 100 μg autologous anti-DNP antibody in IFA, Group 3 received 100 μg autologous anti-DNP antibody in CFA, Group 4 received IFA alone, Group 5 received CFA alone. Control values were the mean of groups 4 and 5.

Figure 11.

The mean relative anti-DNP IgG concentration as measured by RIA. All dogs received DNP-ASC immunization at weeks 0,2,4,6,8 and 10. At weeks 7 and 9, the treatment consisted of immunization with: Group 1, 10 µg autologous anti-DNP antibody in CFA; Group 2, 100 µg autologous anti-DNP antibody in IFA; Group 3, 100 µg autologous anti-DNP antibody in CFA; Control, either IFA or CFA alone. The relative antibody concentration was calculated from an arbitrary antobody concentration scale derived from the titration of a standard anti-DNP IgG containing serum (see text for further details).

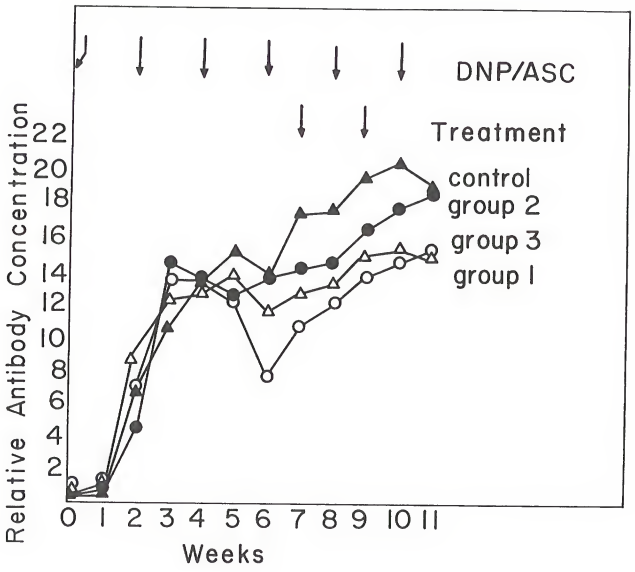

DNP/ASC
Treatment
control
group 2
group 3
group 1
Relative Antibody Concentration
22
20
18
16
14
12
10
8
6
4
2
0 1 2 3 4 5 6 7 8 9 10 11
Weeks

Figure 12.

The mean relative anti-DNP IgE concentration as measured by RIA. All dogs received DNP-ASC immunization at weeks 0,2,4,6,8 and 10. At weeks 7 and 9, the treatment consisted of immunization with: Group 1, 10 µg autologous anti-DNP antibody in CFA; Group 2, 100 µg autologous anti-DNP antibody in IFA; Group 3, 100 µg autologous antibody in CFA; Control, either IFA alone or CFA alone. The relative antibody concentration was calculated from an arbitrary scale derived from the titration of a standard anti-DNP IgE containing serum (see text for further details).

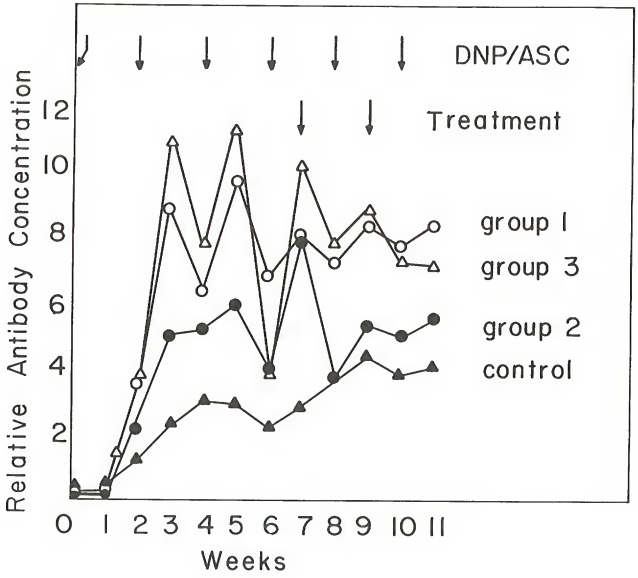

DNP/ASC
Treatment
group 1
group 3
group 2
control
Relative Antibody Concentration
12
10
8
6
4
2
0 1 2 3 4 5 6 7 8 9 10 11
Weeks

Table 9
The Relative Anti-DNP IgE Concentration in 28 Dogs a)

Group 1 b) (10 µg Anti-DNP Antibody in CFA)

Animal Number / Weeks	1	2	3	4
8	2.0±.17	6.4±.29	6.1±.49	8.1±.36
9	2.6±.26	9.3±.84	6.5±.25	9.4±.36
10	3.0±.18	8.7±.31	3.4±.30	7.1±.79
11	2.8±.31	9.3±1.26	4.1±.19	8.2±.81
Animal Number / Weeks	**5**	**6**	**7**	**8**
8	5.2±.57	15.6±.70	6.6±.68	7.2±.30
9	8.9±.63	14.0±.69	6.8±.78	9.2±1.18
10	9.3±.72	13.2±.36	8.2±.14	8.2±.07
11	10.2±.64	15.1±.47	7.4±.66	8.6±.42

Group 2 (100 µg Anti-DNP Antibody in IFA)

Animal Number / Weeks	9	10	11	12
8	9.8±.47	10.0±.22	5.7±.67	5.5±.86
9	9.5±1.89	9.7±.46	7.5±.83	8.5±.33
10	9.8±.67	8.3±.57	4.1±.65	6.8±.41
11	9.4±.59	4.1±.12	5.3±.41	9.3±.17

Group 3 (100 µg Anti-DNP Antibody in CFA)

Animal Number / Weeks	13	14	15	16
8	2.4±.02	3.3±.30	0	4.2±.21
9	4.1±.62	4.0±.50	0	2.9±.14
10	2.0±.39	6.3±.35	0	7.7±.79
11	3.0±.48	9.6±.47	0	2.9±.29
Animal Number / Weeks	**17**	**18**	**19**	**20**
8	6.1±.22	5.6±.49	.6±.01	7.6±.51
9	9.2±.35	8.9±.46	6.0±.43	7.0±.88
10	6.4±.57	5.2±.34	4.5±.06	6.8±.50
11	7.3±.87	6.8±.65	6.4±.06	7.2±1.01

Table 9 Continued

Animal Number Weeks	Group 4 (IFA Alone) 21	22	Group 5 (CFA Alone) 23	24
8	8.0±.69	5.2±.48	2.2±.29	.3±.02
9	9.0±1.22	7.6±.71	2.2±.20	0
10	7.1±.51	7.0±1.06	1.8±.36	0
11	8.5±.34	5.3±.44	2.6±.09	0

Animal Number Weeks	Group 5 Continued 25	26	27	28
8	1.0±.02	4.3±.34	4.2±.44	5.0±.28
9	1.9±.18	4.3±.31	4.2±.35	6.3±.06
10	1.1±.16	3.1±.41	5.7±.26	4.7±.71
11	1.4±.21	2.9±.24	5.2±.38	5.9±.29

a) The level of anti-DNP IgE in these dogs from 0 to week 7 is found in Table 2.
b) Each dog received DNP/ASC immunization at weeks 0,2,4,6,8,10. At weeks 7 and 9: Group 1, 10 µg autologous anti-DNP antibody in CFA; Group 2, 100 µg autologous anti-DNP antibody in IFA; Group 3, 100 µg autologous anti-DNP antibody in CFA; Group 4, IFA alone; Group 5, CFA alone.

Table 10
The Relative Anti-DNP IgG Concentration in 28 Dogs a)

Animal Number / Weeks	Group 1 b) (10 µg Anti-DNP Antibody in CFA) 1	2	3	4
8	9.0±.11	24.7±.45	6.6±.86	8.3±.75
9	9.0±.66	27.4±2.50	10.0±.52	7.5±.78
10	10.2±.93	28.5±.76	10.1±.77	6.2±1.05
11	9.1±1.04	26.8±.39	6.9±.30	9.8±.76

Animal Number / Weeks	Group 1 Continued 5	6	7	8
8	5.5±.23	24.7±1.73	9.4±1.37	17.3±.75
9	4.6±.68	27.0±.39	14.0±.38	20.3±.72
10	5.8±.89	29.1±2.13	14.7±87	17.6±.48
11	8.9±.24	26.6±.90	12.3±.99	19.7±1.29

Animal Number / Weeks	Group 2 (100 µg Anti-DNP Antibody in IFA) 9	10	11	12
8	17.1±1.37	8.9±.76	8.1±.62	14.2±.83
9	20.9±1.47	8.8±.99	9.2±.50	16.4±.38
10	22.9±1.10	11.9±.26	8.7±.61	15.4±1.12
11	25.4±.43	11.3±.69	8.3±.90	15.0±.32

Animal Number / Weeks	Group 3 (100 µg Anti-DNP Antibody in CFA) 13	14	15	16
8	5.6±.17	13.9±.83	7.9±.67	16.7±.99
9	10.3±.62	19.0±.65	11.8±1.12	16.0±.87
10	12.7±.41	20.4±.11	14.3±1.57	15.0±1.01
11	12.2±.73	20.3±2.24	14.7±.59	17.4±2.56

Table 10 Continued

Animal Number Weeks	Group 3 Continued 17	18	19	20
8	7.3±.51	25.5±3.14	24.8±.64	14.6±1.73
9	6.5±.60	21.6±1.19	29.0±2.62	16.8±.96
10	8.4±.62	24.3±.29	30.1±4.71	17.2±2.33
11	9.1±.76	23.1±1.79	30.8±1.06	22.9±1.87

Animal Number Weeks	Group 4 (IFA Alone) 21	22	Group 5 (CFA Alone) 23	24
8	15.6±.93	23.3±1.87	30.1±1.74	8.1±.93
9	16.7±.81	28.7±1.61	27.7±.50	9.5±.62
10	16.2±1.3	31.4±1.05	31.9±3.81	10.7±1.08
11	17.1±.43	30.0±2.62	26.5±2.62	9.6±.27

Animal Number Weeks	Group 5 Continued 25	26	27	28
8	9.3±.81	13.6±.55	20.2±.96	22.2±1.43
9	10.4±.65	18.7±1.17	22.4±.68	23.4±.93
10	11.7±.88	20.5±1.48	17.3±1.24	24.3±.74
11	11.2±.76	18.0±1.42	18.7±.48	22.0±2.38

b) Each dog received DNP/ASC immunization at weeks 0,2,4,6,8,10. At weeks 7 and 9: Group 1, 10 µg autologous anti-DNP antibody in CFA; Group 2, 100 µg autologous anti-DNP antibody in IFA; Group 3, 100 µg autologous anti-DNP antibody in CFA; Group 4, IFA alone; Group 5, CFA alone.

for IgE and IgG for each dog is given in table 9 and 10. There was a gradual increase in the mean antibody concentration in general for both IgE and IgG antibody whereas IgM was not detected after week 6. In two dogs (15, 24), after the seventh and eighth weeks respectively, there was a cessation of the IgE response. Although the mean IgE antibody response increased for the groups in general, individual dogs varied considerably. For example, dogs 14 and 18 had a peak IgE antibody response at week 7 and thereafter the response diminished, whereas the peak response for dogs 19 and 27 occurred at the end of the schedule.

There was no marked difference in the antibody response between the different groups of dogs. To determine if there were any patterns in the antibody response between these groups, an analysis of variance comparing time by group was calculated for each antibody class with the assistance of the Department of Biostatistics, College of Medicine, University of Florida. There was no significant difference between these groups at any given time by this analysis (p greater than .05).

Because of the large variation between dogs, an analysis of variance was calculated comparing dogs within a single litter in one group to dogs from the same litter in other groups as a function of time. This analysis was used to determine if there was variation between one treatment in the IgE or IgG antibody response as compared to a second

treatment within a single litter. In no case was a significant difference observed.

Discussion

The fact that anti-DNP antibody could not be detected in the effluent from the affinity column indicates that the column was effective in removing all anti-DNP antibody. The inability to detect IgE in the glycine eluate was expected because canine IgE is not stable at low pH. Halliwell (7) has shown that at a pH of 2.5 for 30 minutes there is greater than a tenfold decrease in detectable IgE antibody.

There are at least four possible reasons why autologous antibody administration failed to regulate the antibody response in these dogs as had been achieved in laboratory animals. Firstly, in these experiments the dogs had an established antibody response whereas in many of the experimental systems where passive antibody showed regulatory effects on antibody production, a primary or early secondary response was manipulated. It has been shown that it is much more difficult to manipulate a preexisting and established response than to alter a developing one. Secondly, there may be something unique about the regulatory effects of passive antibody on an immune response in young animals. Antibody is transferred from mother to

young both before and shortly after birth. If this passive antibody would result in long term suppression, then it might result in a negative selection process in those animals by rendering them immunologically non-responsive to pathogenic agents. Therefore, very young animals may be less susceptable to the regulatory action of passive antibody. In fact, in a very recent report by Jarrett and Hall (83), they demonstrated that maternal antibody or passively administered antibody given to newborn rats resulted in an enhanced IgG antibody response when challenged with antigen at six weeks of life. However, not every rat so treated had this enhanced IgG response and some rats had a decrease in their IgE response. Thirdly it has been hypothesized that one way in which passive antibody administration could regulate antibody was through the generation of an anti-id response (30). It is possible that any anti-id produced by these dogs was not sufficient to regulate antibody. Lastly, such regulation may result in a clonal escape mechanism. Pawlak et al. (84) has shown that the administration of anti-id to A/J mice against the major cross reactive idiotype produced in these mice immunized to p-azobenzenearsonate would suppress this idiotype and other cross reactive idiotypes, but there was a compensatory increase in other idiotypes not ordinarily expressed in these mice. If the administration of autologous antibody had regulated a subpopulation of antibody

molecules but in response other antibody molecules were expressed, the net effect may not be observable if the entire class specific response were to be measured as was the case in these experiments.

Summary and Conclusions

The administration of autologous anti-DNP antibody in adjuvant to dogs which had an ongoing anti-DNP antibody response did not have a significant effect on this antibody response as compared to control dogs who received adjuvant without autologous antibody. This would suggest either that the regulation by passive antibodies, as seen in laboratory animals, does not operate in this species or that any regulation that occurred by such treatment could not be detected by the methods used in this study.

CHAPTER FOUR
THE IDENTIFICATION OF ANTI-IDIOTYPIC ANTIBODY

Introduction

Anti-idiotypic antibody has been produced by immunizing an animal with isologous or autologous antibody in adjuvant (40-42). The use of isologous or autologous antibody rather than homologous antibody eliminates the potential that allotypic determinants might be recognized rather than idiotypic determinants. The immunization schedule used in the previous experiments included the administration of autologous antibody in adjuvant. It was hoped that this treatment would regulate IgE antibody, but unfortunately, it did not. It was not known if this failure was because of a lack of an anti-id response or for other reasons. This treatment may have induced anti-id. It is also possible that anti-id may have been produced during the immunization with antigen. The purpose of the experiments in this chapter was to determine if, at any time during the immunization schedule, anti-id was detectable.

Materials and Methods

Antisera

The antisera used in these experiments were described in Chapter two. Any cross reactive anti-mouse immunoglobulin activity that was present in the anti-canine IgG, IgM or IgE antisera used in the anti-id RIA was removed by passage through an affinity column which had bound to it a 40 percent saturated ammonium sulfate precipitate of normal mouse serum.

Animals and Immunization

The animals and immunization schedules have been described in Chapter two and three except that in the experiment designed to determine if the specificity of the antibody was important in the induction of an anti-id response, a different immunization protocol was used. Eight mature dogs were injected with 100 µg of aluminum hydroxide precipitated DNP-ASC by the intraperitoneal route on the day of arrival. At the same time, these dogs received a second injection of 100 µg of aluminum hydroxide precipitated ABA-KLH by the same route at a different site. These dogs were immunized three times at two week intervals. At the time of the last immunization, 30 ml of blood were obtained from each dog. The serum from this blood was used to purify antibody. The dogs were

arbitrarily placed into one of three groups. Group 1 (N=3) received 100 μg of autologous anti-DNP antibody in CFA by the subcutaneous route. This antibody was purified from serum by adsorption to and elution from a DNP coupled affinity column followed by passage through an ABA coupled affinity column to ensure that the purified anti-DNP antibody had no cross reactive anti-ABA antibody. Conversely, the autologous anti-ABA antibody in CFA for Group 2 (N=3) was purified from serum by adsorption to and elution from an ABA affinity column followed by passage through a DNP column. The control group, Group 3 (N=2) received CFA without autologous antibody. This later immunization was administered six weeks after the primary injection of antigen. Two weeks after this last injection, serum from each animal was assayed for anti-idiotypic antibody.

Anti-id RIA

The RIA used to detect anti-id was performed essentially as described for the antigen specific RIA using a number of mouse monoclonal anti-DNP antibodies as the antigen. These were

a) Anti-DNP IgG, (a gift from Dr. A.P. Lopes, University of Pennsylvania) and as a control antibody, anti-H_2K IgG (a gift from Dr. P. Klein, University of Florida).

b) Anti-DNP and IgE as a control antibody, anti-OVA IgE.

c) Anti-DNP IgM and as a control antibody, anti-SRBC IgM.

The antibodies b) and c) were obtained from Sera-Labs, Accurate Chemical and Scientific Corp., Westbury, N.Y.

d) Anti-DNP IgM (a gift from Dr. C.W. Clem, Mississippi State University. This antibody will be designated anti-DNP $IgM-_2$), and as a control antibody, anti-SRBC IgM (a gift from Dr. W.C. Raschke, La Jolla Cancer Research Foundation, Ca.).

The wells were coated with 10 µg antibody in 50 µl of Tris buffer (0.1 M, pH 8.0). A radiolabelled anti-canine IgG antibody was used as the radiolabelled probe unless otherwise stated.

Hapten Inhibition of Id/anti-id Interaction

The RIA using mouse anti-DNP IgG monoclonal antibody or control IgG monoclonal antibody was performed as previously described except that after blocking any remaining active sites by the addition of HSA to the plates, various amounts of 2,4-dinitrophenol glycine (Sigma Chemical Co., St. Louis, Mo.) ranging from 0.001 to 0.1 mg in PBS were incubated for three hours at $4^{o}C$. Serum samples were then added to the wells, incubated for three hours at $4^{o}C$ and washed to remove unbound protein. A radiolabelled anti-canine IgG antibody was added, incubated for three

hours at 4°C and the wells were washed to remove unbound radiolabelled antibody. The amount of radioactivity associated with the well was determined in a gamma counter.

$$\% \text{ Inhibition} = \frac{\text{cpm sample in the presence of hapten}}{\text{cpm sample in the absence of hapten}} \times 100$$

Inhibition of Antigen Antibody Interactions by Anti-Idiotypic Antibodies

The RIA using mouse anti-DNP IgG monoclonal antibody or a subtype and allotype matched control mouse IgG monoclonal antibody was performed except that after blocking remaining active sites by the addition of HSA to the plates, serum with or without anti-id was added, incubated for three hours at 4°C and washed three times with RAST-buffer. Antigen (125 I DNP-HSA, approximately 20,000 cpm) was added to the wells and incubated for three hours at 4°C and each well was washed five times to remove unbound antigen. Any anti-id that was bound to the anti-DNP antibody may inhibit this antigen-anti-DNP interaction. The amount of antigen bound to the wells was determined in a Packard gamma counter. The percent inhibition by anti-id was calculated by

$$\% \text{ Inhibition} = 1 - \frac{\text{cpm bound to plates after serum incubation}}{\text{cpm bound to plates after PBS incubation}} \times 100$$

Results

Identification of Canine Anti-Iditoypic Antibody

A screening procedure was used to assay for the presence of anti-id in serum obtained during the immunization schedule. Those dogs that received autologous antibody produced an antibody which would bind to mouse monoclonal anti-DNP IgG as seen in table 11. This binding was not detectable prior to such treatment in any dog nor could it be detected in control dogs at any time. There was no detectable binding to the anti-H_2K IgG mouse antibody in serum from any dog.

The experiment was repeated with another group of 12 dogs and the anti-id activity was converted to an arbitrary relative antibody concentration by interpolation from a scale derived from the titration of a positive high titer sample identified in the screening procedure (table 12). This standard serum was given a relative antibody concentration of 10. Some dogs produced detectable levels of this anti-idiotypic antibody within one week after autologous antibody administration whereas other dogs took three weeks to develop such a response. There was also variation in the magnitude of the response observed. This anti-idiotypic antibody could not be detected if anti-canine IgE or IgM antisera was used as the radiolabelled probe rather

Table 11
Screening for Canine IgG Anti-Idiotypic Antibody by RIA
Using Mouse Monoclonal Anti-DNP IgG as the Antigen

Animal # a)	2	3	4	6	14
Week					
0	476±16	331±19	448±7	397±14	422±21
1	453±7	590±1	481±11	421±10	470±47
2	412±21	347±3	274±3	335±6	443±15
3	317±9	277±11	352±9	371±3	367±40
4	305±15	358±3	367±27	401±16	457±12
5	378±31	376±7	358±18	421±11	352±25
6	396±14	417±37	284±10	379±23	318±14
7	421±27	335±10	409±15	522±27	314±24
8	1357±31	1815±26	433±21	2173±68	277±43
9	2140±150	2027±36	1533±117	2250±137	1420±48
10	1862±17	2208±55	2092±130	2297±100	2135±186
11	2174±79	3058±121	2107±41	3375±46	5087±161

Animal #	17	19	20	23	24
Week					
0	476±1	367±40	307±22	422±9	483±19
1	453±17	351±26	406±19	470±16	517±42
2	418±5	481±56	464±3	394±7	347±5
3	519±43	435±13	253±13	367±14	318±23
4	442±16	467±24	373±20	334±17	351±1
5	376±18	430±45	481±6	442±26	235±7
6	340±28	398±1	295±30	462±11	434±16
7	315±7	384±93	563±38	371±31	346±34
8	798±13	1937±179	721±37	529±21	471±18
9	973±10	2476±88	936±68	315±8	512±46
10	1611±86	2720±9	2437±177	447±60	341±28
11	1735±122	2925±66	2026±69	473±3	429±27

a) All dogs were immunized with DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10. At weeks 7 and 9, dog 2,3,4 and 6 received 10 µg autologous anti-DNP antibody in CFA; dogs 14,17,19 and 20 received 100 µg autologous anti-DNP antibody in CFA; dogs 23 and 24 received CFA alone.

b) This represents the mean ± standard deviation of a triplicate sample. All samples were assayed at a serum dilution of 1/5 in PBS.

The mean ± standard deviation for all samples assayed using mouse anti-H_2K IgG_1 was 477±97.

Table 12
The Relative Antibody Concentration of Canine IgG Anti-Idiotypic Antibody as Measured in a RIA using Mouse Monoclonal Anti-DNP IgG as the Antigen a)

Animal Number / Weeks	1	5	7	8	13
0	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	3.1±.2	4.0±.2	0	0	0
9	5.3±.4	5.4±.4	3.1±.3	3.1±.4	0
10	4.3±.3	5.1±.4	3.8±.2	5.4±.6	3.4±.2
11	5.2±.4	8.0±.6	2.9±.1	5.5±.2	6.6±.4

Animal Number / Weeks	15	16	18	26	27
0	0	0	0	0	0
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	0	2.4±.1	1.2±.1	0	0
9	3.7±.2	2.2±.2	2.8±.1	0	0
10	10.0±1.3	4.3±.3	5.2±.4	0	0
11	9.2±.7	3.3±.1	7.1±.3	0	0

a) The relative antibody concentration ± range was determined by interpolating from the titration of a serum sample containing high levels of anti-id. A value of zero indicates no detectable anti-idiotypic antibody.
b) All animals received DNP-ASC adjuvant at weeks 0,2,4,6,8,10. At weeks 7 and 9 all animals received autologous antibody in adjuvant except 26 and 27 who received adjuvant alone.

than the anti-IgG antisera, indicating that it was of the IgG class.

When three different anti-DNP monoclonal antibodies and three control monoclonal antibodies were used, the binding activity was detected only with the original anti-DNP IgG (table 13), and to a lesser extent, to anti-DNP $IgM-_2$ (table 14). In those animals in which anti-DNP $IgM-_2$ binding activity was detected, a comparison was made between the serum from a point in time prior to autologous antibody administration and serum obtained after such treatment. A minimum value that was two standard deviations above the mean of the control was considered indicative of anti-id activity. As was the case with the IgG antibody, only those animals which received autologous antibody, showed binding activity and only after administration of autologous antibody (table 14).

The Role of Antibody in the Specificity of the Anti-Idiotypic Production

To determine if immunization with an antibody whose specificity was other than anti-DNP would result in anti-DNP/anti-id, eight dogs were given both DNP/ASC and ABA/KLH three times at two week intervals. Six weeks after the primary injection of antigen, three dogs received 100 μg of autologous anti-DNP antibody in CFA, and a different three dogs received 100 μg autologous anti-ABA antibody in

Table 13
Detection of Canine IgG Anti-Idiotypic Antibody
by RIA With Various Mouse Monoclonal Antibodies

Animal #	Anti-DNP (IgE)	Anti-OVA (IgE)	Anti-DNP (IgM)
26	234±7	267±25	264±4
27	281±1	335±89	151±6
16	171±3	286±48	286±3
15	321±31	261±31	301±29
8	261±13	284±14	322±17
7	287±69	221±8	361±37
13	389±11	205±34	257±28
	108±45	105±34	

Animal #	Anti SRBC (IgM)	Anti-DNP (IgG)	Anti-ABA (IgG)
26	182±36	321±95	209±47
27	197±12	354±29	218±38
16	176±42	4777±65	207±7
15	106±7	7571±246	176±12
8	106±7	1273±93	178±31
7	114±8	980±47	168±14
13	189±15	1144±68	221±8

Serum was diluted 1:2 in PBS. All samples were from week 10 of the immunization schedule. All animals had received DNP-ASC in adjuvant at week 0,2,4,6,8,10 and at weeks 7 and 9 received autologous antibody in CFA except 26 and 27 who received CFA alone.

Table 14
Detection of Canine IgG Anti-Idiotypic Antibody by RIA with Mouse Monoclonal Anti-DNP IgM_2 Antibody

Dilution of Serum	Anti-DNP IgM_2 Antibody					
	26-E[a]	26-L	27-E	27-L	16-E	16-L
1/5	162±11	286±21	135±14	248±45	90±7	643±31
1/10	82±14	154±15	97±26	152±29	86±	296±19
1/20	71±21	135±9	126±36	181±11	103±11	156±12
	15-E	15-L	8-E	8-L	7-E	7-L
1/5	225±31	912±25	216±47	570±46	249±1	672±6
1/10	184±8	442±27	128±6	373±31	125±8	343±37
1/20	115±12	247±6	102±25	270±12	86±7	236±24
	5-E	5-L				
1/5	128±33	741±54				
1/10	106±7	358±54				
1/20	134±8	229±11				

a) The E indicates serum obtained prior to the administration of autologous antibody administraion (16,15,8,7) or adjuvant alone (26,27), at week 6.
The L indicates serum obtained after such treatment from week 10.
The mean ± standard deviation for all samples assayed using anti-SRBC IgM was 186±53.

CFA. Two dogs received CFA alone. Serum before and two weeks after this treatment was screened for anti-id activity. As seen in table 15, the dogs that received autologous anti-DNP antibody produced an anti-id which was detected by the anti-DNP IgG mouse monoclonal antibody and failed to bind to the anti-ABA IgG. Dogs that received anti-ABA antibody in adjuvant developed anti-id which bound to anti-ABA mouse monoclonal IgG but failed to bind to the anti-DNP IgG mouse monoclonal antibody. The two control dogs produced no detectable anti-id that was reactive with either mouse monoclonal antibody.

Hapten Inhibition and Elution Studies of the Id/anti-id Interaction

The anti-idiotypic RIA was used to determine if hapten could inhibit the binding of anti-id to the mouse anti-DNP antibody. In two of the six cases (5,15), 10 μg of hapten was able to inhibit id/anti-id interaction (table 16) as shown by a slight decrease in the cpm bound to the wells as compared to the same sample incubated with PBS (22 percent and 26 percent inhibition respectively). As the concentration of hapten decreased, so did the percent inhibition, (17 percent and 5 percent at a fivefold decrease in hapten concentration). However, it is unclear how significant this inhibition was because of the extremely large amounts of hapten required to obtain these

results. Similarly, hapten was unable to elute anti-id from id when a concentration of DNP-glycine of up to 20 µg was used.

Inhibition of Antigen Binding to Antibody by Anti-Idiotypic Antibody

Since hapten could not consistently interfere with id/anti-id, it was reasoned that perhaps a large hapten coupled molecule might interfere with this interaction. Although the anti-id was not binding to id determinants within the antigen binding site, it may have bound to determinants close enough to the hypervariable region of the antibody molecule to sterically hinder antigen binding to antibody. Serum containing anti-id was preincubated with anti-DNP mouse monoclonal antibody prior to the addition of a radiolabelled DNP-HSA antigen to determine if the presence of anti-id could inhibit the anti-DNP antibody DNP-HSA antigen interaction. As seen in table 17, anti-id was able to inhibit the binding of the radiolabelled antigen to the mouse monoclonal antibody. Animal 26 had no detectable anti-id, and serum from this dog failed to interfere with antigen binding to antibody. In contrast, the other dogs had detectable anti-id and inhibited this interaction from 21 to 50 percent of the maximum cpm bound.

Table 15
Specificity of Canine IgG Anti-Idiotypic Antibody
After the Administration of
Autologous Antigen-Specific Antibody

		Anti-ABA Group 1 a)			Anti-DNP Group 2 b)	
		Antibody in well			Antibody in well	
Animal #	Serum Dil.	Anti-DNP IgG_1	Anti-ABA IgG_1	Animal #	Anti-DNP IgG_1	Anti-ABA IgG_1
29	1/5	391±36	1901±101	33	1243±37	134±53
	1/10	236±13	1675±118		996±115	142±13
	1/20	153±14	1145±141		703±10	249±86
30	1/5	293±114	1867±157	34	1221±101	113±65
	1/10	186±26	1383±57		683±6	261±69
	1/20	85±37	1061±46		545±59	143±19
31	1/5	383±35	1070±96	35	902±36	317±29
	1/10	248±89	899±18		531±106	131±16
	1/20	103±15	494±86		331±21	129±21
Control c)						
32	1/5	172±16	246±42	36	238±33	186±26
	1/10	158±47	117±28		186±32	133±58
	1/20	98±8	88±14		121±13	94±17

Each dog received three immunizations with ABA-KLH and DNP-ASC at two week intervals. Six weeks after primary immunization, dogs received either.

a) Group 1 received 100 µg autologous anti-ABA antibody in CFA
b) Group 2 received 100 µg autologous anti-DNP antibody in CFA
c) Control received CFA alone
d) Mean cpm of the sample assayed in triplicate ± standard deviation. The serum was diluted 1/5 with PBS.

The serum used in this assay was obtained 2 weeks after this later immunization.

Table 16
The Inhibition of Canine IgG Anti-id Binding to
Mouse Monoclonal Anti-DNP IgG by Hapten
as Measured by RIA

	Animal Number a)		
2, 4 DNP Glycine	1	5	7
0 µg	3943 ± 73 b)	4522 ± 138	1775 ±34
10 µg	3802 ± 100	3533 ± 69	1704 ± 91
2 µg	3983 ± 280	3783±136	1675 ± 46
1 µg	3807 ± 214	4268 ± 56	1614 ± 101
0.1 µg	3804 ± 12	4623 ± 219	1734 ± 73
	8	15	16
0 µg	2281 ± 162	2702 ± 131	1643 ± 52
10 µg	2203 ± 200	2026 ± 168	1454 ± 15
2 µg	2012 ± 115	2593 ± 173	1691 ± 14
1 µg	2148 ± 83	2738 ± 57	1377 ± 34
0.1 µg	2213 ± 129	2694 ± 89	1526 ± 31

a) Each dog was immunized with autologous anti-DNP antibody in CFA at weeks 7 and 9. All samples were from week 9 in the schedule except sample 15 which was from week 11.
b) This is the mean cpm of triplicate samples ± standard deviation. All samples were assayed with serum diluted one to four with PBS.
A control well with mouse anti-H_2K IgG_1 rather than anti-DNP IgG was assayed for each sample. The mean ± standard deviations for all samples 283±47.

Table 17
Inhibition of binding of 125 I-DNP/HSA to
Mouse Monoclonal Anti-DNP Antibody
by Canine Anti-Idiotypic Antibody

Animal a)	C.P.M. Bound + S.D. b)	% Inhibition c)
26	994 ± 43	0
8	493 ± 38	50
18	544 ± 25	45
13	783 ± 41	21
15	611 ± 13	39
1	521 ± 18	48

a) Animal 8, 18, 13, 15 and 1 received autologous antibody in CFA and had detectable levels of anti-id; animal 26 received CFA alone and did not have detectable anti-id. all serum was obtained at week 11.
b) This is the mean cpm ± standard deviation of a sample assayed in triplicate
c)

$$\% \text{ Inhibition} = \frac{\text{c.p.m. control} - \text{c.p.m. sample}}{\text{c.p.m. control}}$$

The control was a set of wells coated with mouse anti-DNP IgG and incubated with PBS rather than serum. The value for this was 1026±19.

Discussion

Dogs immunized with autologous antibody produced an antibody which bound to one anti-DNP mouse monoclonal IgG. This antibody was present only after such treatment and not present prior to the administration of autologous antibody. The specificity was limited to id determinants present on some but not all anti-DNP mouse monoclonal antibodies. It could not be detected using mouse monoclonal antibodies whose specificity was other than DNP such as anti-H_2K IgG or anti-ABA IgG. This putative anti-id had no specfificity for mouse immunoglobulin heavy or light chain constant region determinants as indicated by the failure to detect any activity when an allotype and isotype match non-anti-DNP antibody was used in the assay as antigen. These findings suggested that this mouse binding protein was anti-idiotypic in nature.

When the serum which contained this anti-id was assayed to determine if this antibody could bind to other mouse anti-DNP monoclonal antibodies, there was no detectable binding to two of the mouse anti-DNP monoclonal antibodies and a limited binding to the monoclonal anti-DNP IgM antibodies. The difference in the level of anti-id detected when either the anti-DNP IgG or the anti-IgM antibody was used as the antigen indicates the difference in

the ability of the anti-id to bind to these two antigens. This difference could be a function of 1) different idiotypic determinants present on the two antibodies, or, 2) the difference in the accessability of the id to the anti-id or 3) a combination of both of these.

Id/anti-id interactions can, in many cases, be inhibited by hapten. If the interaction is hapten inhibitable, it suggests that anti-id binds to id determinants within the antigen combining portion of an antibody molecule or to idiotypes intimately associated with this region. In those instances where hapten is unable to inhibit this interaction, it can be concluded that anti-id is binding to those ids not within the antigen binding site. It also indicates that anti-id does not act as an internal image of antigen. High concentrations of hapten relative to the amount of antibody on the plate were preincubated with mouse anti-DNP IgG antibody. The presence of hapten did not consistently inhibit canine anti-id/mouse id interaction. Only two of the six samples tested showed inhibition, with 27 percent being the maximum inhibition. In other experiments, hapten could not displace the anti-id from the mouse anti-DNP antibody. These results suggest that the majority of anti-id is not binding to structures within the antigen binding site of the mouse monoclonal anti-DNP IgG. An anti-id and an internal image of antigen both bind to structures within the variable regions of an

antibody molecule. However, an internal image of antigen binds to the hypervariable regions associated with the antigen combining site.

If this antibody was an internal image of antigen then the activity should have been detected when each anti-DNP antibody was used as the antigen. Also, a hapten should inhibit the binding of an internal image of antigen to the respective antibody. Since anti-id bound to only two anti-DNP mouse monoclonal antibodies and the id-anti-id interactions were not consistently inhibited by hapten, it can be concluded that this anti-id is not an internal image of antigen.

Although hapten could not inhibit the id/anti-id interactions in all cases, indicating that the recognized idiotopes were not within the antigen combining sites, these id may be very close to the antigen combining site. Serum which contained anti-id was assayed to determine if the sample could interfere with the interaction between the mouse anti-DNP antibody and a radiolabelled dinitrophenylated antigen. In all samples containing anti-id, the level of antigen bound to the mouse antibody was decreased, although complete inhibition of this binding was not observed. Anti-id could consistently inhibit antibody/-antigen interactions. However, the id/anti-id interactions were not hapten inhibitable. Therefore, some of these anti-ids must bind to id determinants which are close to

the antigen combining site and other anti-ids bind to ids that are more distant. This failure to observe complete inhibition could have been for at least two reasons. There was not sufficient anti-id in the serum to block all the antigen binding sites. Alternatively, the anti-id bound to id determinants located on the molecule in such a way that complete inhibition of the antigen binding site was not possible.

The results in this chapter show that those animals given autologous antibody in adjuvant produced an anti-id. This anti-id response is not a function of the administration of adjuvant because control dogs given adjuvant without antibody failed to produce detectable levels of anti-id. In those dogs that produced anti-id the autologous antibody that was used for immunization had been subjected to harsh treatment (e.g. glycine HCl elution from an affinity column) during the purification process. This treatment could possibly alter the ids present in the antibody. Therefore it might be possible that the antigen specificity of the antibody has little or nothing to do with the anti-id that is produced.

To address this question, dogs were immunized to two different antigens, DNP-ASC and ABA-KLH. They were then given either autologous anti-DNP antibody or autologous anti-ABA antibody emulsified in adjuvant and subsequently produced an anti-id which bound to mouse monoclonal

antibody of the same specificity as the immunizing antibody. Prior to such treatment this autologous antibody is present in the dog but does not induce detectable levels of anti-id. However, after the administration of this same antibody, anti-id is detected. Therefore, these results indicate that during the purification procedure and/or the immunization procedure, anti-id determinants present on the autologous antibody are immunogenically enhanced. However, any changes that occur in the protein molecule must be subtle because the specificity of the anti-id response was determined by the specificity of the immunizing antibody. If there was marked change in the id determinants, then the anti-id might not be expected to maintain its specificity for the immunizing antibody. However, it is not possible from these results to determine if the immunogenic enhancement of the id was a result of the purification process, the route of immunization or a combination of both these things.

It was very fortunate that the mouse antibody used as the antigen in these assays had id determinants which could bind to the canine anti-id, although shared idiotypy between animals has been reported (53,57,85-87).

Summary

The results in this chapter suggest the following: Anti-id can be induced by the administration of autologous antibody. The majority of this anti-id is not hapten inhibitable and is detectable with only a few monoclonal antibodies of the same specificity which presumably bear the same or a similar set of cross reactive idiotypes. Furthermore, this anti-id is not an internal image of antigen.

Conclusions

1) The administration of autologous antibody in adjuvant induces a reciprocal anti-idiotypic antibody response. 2) The identification of these anti-id antibodies was achieved by the use of monoclonal anti-DNP antibody from another species as a source of idiotype.

CHAPTER FIVE
DETECTION OF ANTI-IDIOTYPIC ANTIBODY USING AUTOLOGOUS ANTI-DNP F(ab)'2 FRAGMENTS AS THE IDIOTYPIC ANTIGEN

Introduction

Anti-idiotypic antibody, as noted in Chapter one, can have a regulatory function during an immune response. It can either enhance (62) or suppress (84,88) the level of the corresponding idiotype. Even if anti-id stimulates only a limited number of ids, the overall result is an enhancement in the total antigen-specific antibody response (63). Anti-id that acts as an internal image of antigen can stimulate or enhance an immune response in a way analagous to antigen (89). On the other hand, anti-id has been shown to suppress an entire antigen-specific isotype (60-61). If anti-id is important as a natural means to regulate antigen-specific antibody, it would seem logical that anti-id would be detectable during a normal immune response. The results of Chapter four indicated that after

immunizing dogs with autologous anti-DNP antibody, anti-id was detected. However, this anti-id was not detected during the response to DNP-ASC.

The failure to detect anti-id prior to the immunization with antibody in adjuvant could be because; 1) anti-id was not present or, 2) the method used to detect it was wrong. The purpose of the experiments in this chapter were to determine if anti-id could be detected at any time during a DNP specific antibody response using autologous anti-DNP $F(ab)'_2$ antibody fragments as the source of id.

Materials and Methods

Preparation and Immobilization of Anti-DNP $F(ab)'_2$ Fragments to a solid Matrix

Anti-DNP $F(ab)'_2$ fragments were prepared from antibody purified from a single serum sample by affinity chromatography. The protein was digested with pepsin and the $F(ab)'_2$ was separated from intact antibody and Fc fragments as previously described in Chapter two. The $F(ab)'_2$ from each sample was handled separately and $F(ab)'_2$ from a single sample will be referred to as a set. Each set of antibody fragments was bound to a solid support matrix (Immunobead^r. BioRad Laboratories, Richmond, CA) as described by the manufacturer. Briefly, a given quantity of anti-DNP $F(ab)'_2$ in 0.003 M

$KH_2(PO_4)$ buffer, pH 6.3 and a proportionate amount of beads were incubated together for one hour at 4^oC followed by the addition of 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide HCl (EDAC) with an additional incubation at 4^oC for one hour. Any remaining active sites were blocked with 1 percent HSA in 0.005 phosphate buffer, pH 7.2 by incubating this with the beads for one hour at room temperature. The beads were pelleted by centrifugation at 1,000 x g for 10 minutes at 4^oC and alternately washed with PBS, pH 7.2 followed by 1.4 M NaCl-PBS, pH 7.2 three times to remove unbound protein. After the final wash the beads were suspended in RAST+ buffer. The beads used in a single experiment were standardized for both DNP-HSA binding and total $F(ab)'_2$ content by incubating an aliquot from each bead set with various dilutions of ^{125} I DNP-HSA or ^{125} I anti-canine light chain antibody. For example, 50 µg of one bead set bound 5,656 $\pm$ 61 cpm radiolabelled anti-canine light chain specific antibody (this number of cpm is approximately 140 ng of anti-canine light chain antibody) and 1,040 $\pm$ 53 cpm radiolabelled DNP-HSA. A second set bound 4,690 $\pm$ 79 cpm anti-light chain antibody (this is approximately 110 ng of anti-canine light chain antibody). and 747 $\pm$ 21 cpm antigen. The second set of beads had approximately 75 percent of the binding capacity of the first set. Therefore 63 µl of beads from the second set were used in

the assay and 50 µl of the first. No immuno-reactive Fc material was detectable on any bead set when ^{125}I anti-canine heavy chain specific IgG was incubated with an aliquot of each bead set.

Detection of Natural Occuring Anti-id

Anti-DNP $F(ab)'_2$ fragments from a single serum sample immobilized as described above was used as an antigen to detect anti-id. Various serum samples from the same dog were assayed for anti-id after being chromatographed through a DNP-affinity column to remove anti-DNP antibody which theoretically could compete by binding anti-id. Each sample was concentrated by negative pressure dialysis to approximately the starting volume of serum. The samples were assayed by incubating an undilute, a 1/2 and a 1/4 dilution in PBS of each sample with a standardized amount of autologous anti-DNP $F(ab)'_2$ bound beads for three hours at room temperature. The beads were then centrifuged and the supernatant removed and washed with RAST+ three times to remove unbound antibody. Radiolabelled heavy chain specific anti-canine IgG was added to each set of beads (approximately 30,000 cpm/sample), incubated for three hours at room temperature and washed to remove unbound radiolabelled antibody. The radioactivity of each sample was determined in a Packard gamma counter. Included in each assay at all sample dilutions were beads

bound with normal canine IgG $F(ab)'_2$ (with no detectable anti-DNP activity) and with HSA bound beads. Specific binding was calculated by using the following formula: specific binding = cpm bound to autologous anti-DNP $F(ab)'_2$ beads of a sample at a given dilution - cpm bound to NCS IgG $F(ab)'_2$ beads of the sample at the same dilution. Since beads were standardized for an amount of antibody in each experiment, the volume of beads used ranged from 50 µl to 78 µl per sample. When 50 or 100 µl of non-specific $F(ab)'_2$ was incubated with the sample, there was less than a 15 percent difference in the cpm indicating that the increase in bead volume had little influence on the background activity.

Results

Identification of Anti-Idiotypic Antibody Using Autologous Idiotypes

The purpose of these experiments was to determine if autologous id could be used to detect anti-id. Anti-DNP $F(ab)'_2$ fragments from various time points in the immunization schedule were used as antigens to detect anti-id in autologous serum. The autologous serum used in these experiments were first chromatographed through a DNP affinity column to remove anti-DNP antibody. This was done to eliminate any possible interference the presence of this

antibody might have. Anti-id was detected during the DNP-ASC immunization schedule in three of the five dogs tested (tables 18-20) but no anti-id was evident in the other two dogs. The kinetics and the amount of anti-id varied depending upon what set of ids were used an antigens and which serum sample was tested. Three different patterns in the appearance of anti-id are seen: Pattern one: Anti-id could not be detected before or coincident with the id but could be detected later, as was seen with two samples in two dogs (figure 13,14). In dog 14, the ids used to detect anti-id were from week two, anti-id was not detected until week seven (figure 13). Similarly, in dog 1, when the ids from week four were used as antigens, anti-id was not detected until week six (figure 14). Pattern two was seen in three dogs using six serum samples. In a single sample, id and anti-id were both present (figure 15-20). For example, when the antibody obtained at week six was used as an id antigen, anti-id was detected at week six but the maximum level of anti-id was later than week six (figure 15). In two samples assayed, the highest level of anti-id was detected from the same samples that were used to obtain the antibody which was used as the id antigen (figures 19 and 20). Pattern three: In dog 14, when the ids which were used as antigens to detect anti-id were from blood obtained at week 11, anti-id was detected with each sample tested (figure 21). Similarly, by using

Table 18
The Detection of Canine Anti-Idiotypic Antibody
by RIA Using Autologous Anti-DNP $F(ab)'_2$ as the Id

Dog Number 1

Source of id (Week) b)	Effluent Dilution	Source of Anti-id (Week) a) 2	4	6	8	11
4	0	82±64	12±12	1332±112	2035±279	150±48
	1/2	41±70	51±38	939±99	1519±199	286±45
	1/4	63±49	0	171±34	1069±170	14±18
6	0	37±31	193±39	611±50	1241±179	1586±42
	1/2	14±13	86±55	534±37	370±27	1333±26
	1/4	10±21	3±5	58±35	12±25	413±53
7	0	3±5	253±26	455±37	1496±14	1300±17
	1/2	43±12	279±121	397±61	534±29	714±51
	1/4	38±27	21±28	179±41	30±21	346±15
10	0	51±25	426±41	556±47	595±257	459±101
	1/2	14±23	349±153	438±32	437±89	257±57
	1/4	150±63	79±71	214±83	139±81	179±68

	Control c)
0	186±9
1/2	149±31
1/4	138±46
	101±7

a) Serum from different times during the immunization schedule.
b) The id was autologous anti-DNP $F(ab)'_2$ immobilized to a solid matrix. The dog was immunized with DNP-ASC in adjuvant at week 0,2,4,6,8 and 10 and received 10 µg autologous anti-DNP antibody in CFA at weeks 7 and 9.
c) Control id was normal canine IgG $F(ab)'_2$ immobilized to a solid matrix.

Table 19
Detection of Canine Anti-Iditoypic Antibody by RIA
Using Autologous Anti-DNP $F(ab)'_2$ as the Id

Dog Number 14

Source of id (Week) b)	Effluent Dilution	Source of Anti-Id (Week) a) 2	4	7	11
2	0	137±31	65±21	644±68	705±18
	1/2	36±18	7±7	152±43	351±47
	1/4	0	5±7	12±11	90±15
5	0	216±39	318±49	381±68	848±9
	1/2	135±23	37±31	150±62	677±34
	1/4	77±64	0	47±15	300±23
11	0	624±5	778±18	838±52	405±77
	1/2	309±7	653±28	786±38	233±16
	1/4	99±29	386±16	493±14	83±11
		Control c)			
	0	321±37			
	1/2	186±7			
	1/4	128±29			

a) Serum from different times during the immunization schedule.
b) The id was autologous anti-DNP $F(ab)'_2$ immobilized to a solid matrix. The dog was immunized with DNP-ASC in adjuvant at weeks 0,2,4,6,8, and 10 and received 100 µg autologous anti-DNP antibody in CFA at weeks 7 and 9.
c) Control id was normal canine IgG $F(ab)'_2$ immobilized to a solid matrix.

Table 20
The Detection of Canine Anti-Iditoypic Antibody by RIA
Using Autologous Anti-DNP $F(ab')_2$ as the Id

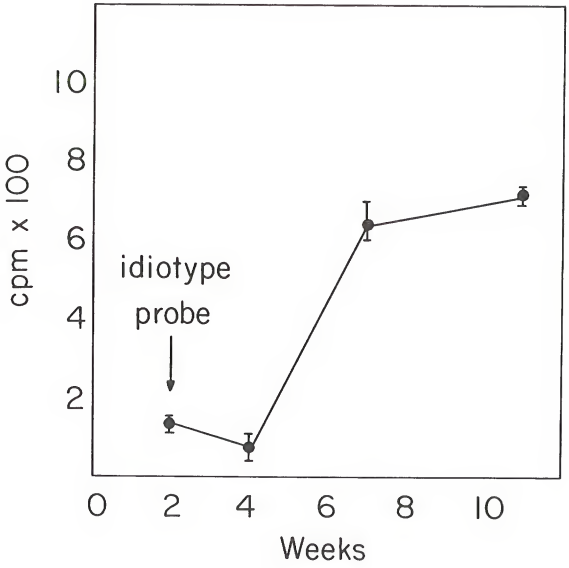
Dog Number 21

Source of id (Week) b)	Effluent Dilution	Source of Anti-Id (Week) a) 1	3	5	7	10
1	0	358±94	1000±49	307±69	435±146	684±33
	1/2	247±37	539±97	204±39	197±55	377±36
	1/4	88±23	102±61	35±7	27±26	246±29
3	0	253±63	1532±88	1031±63	299±27	31±28
	1/2	77±31	865±109	940±39	5±3	150±123
	1/4	83±71	276±123	220±47	2±4	121±38
7	0	104±60	0	0	1466±229	1077±10
	1/2	0	0	0	364±97	591±101
	1/4	46±29	0	75±50	169±11	359±66
11	0	49±41	0	89±31	0	0
	1/2	0	65±58	28±49	69±63	64±57
	1/4	108±39	17±11	21±43	101±34	106±29
		Control c)				
	0	238±83				
	1/2	211±61				
	1/4	103±48				

a) Serum from different times during the immunization schedule.
b) The id was autologous anti-DNP $F(ab')_2$ immobilized to a solid matrix. The dog was immunized to DNP-ASC in adjuvant at weeks 0,2,4,6,8, and 10 and received CFA alone at weeks 7 and 9.
c) Control id was normal canine IgG $F(ab')_2$ immobilized to a solid matrix.

Figure 13.

The identification of anti-id in various serum samples over time in dog 14 as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and autologous anti-DNP antibody in adjuvant at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.


cpm x 100
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idiotype
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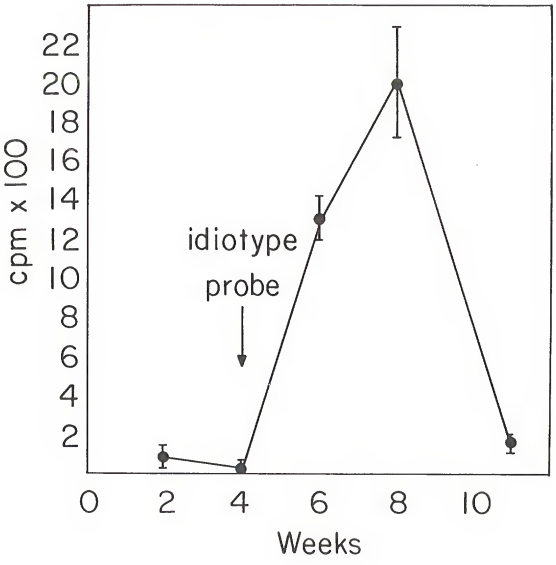
Weeks

Figure 14.

The identification of anti-id in various serum samples over time, in dog 1, as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10 and autologous antibody in adjuvant at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigen to detect anti-id. The bars represent the standard deviation of the mean.


cpm x 100
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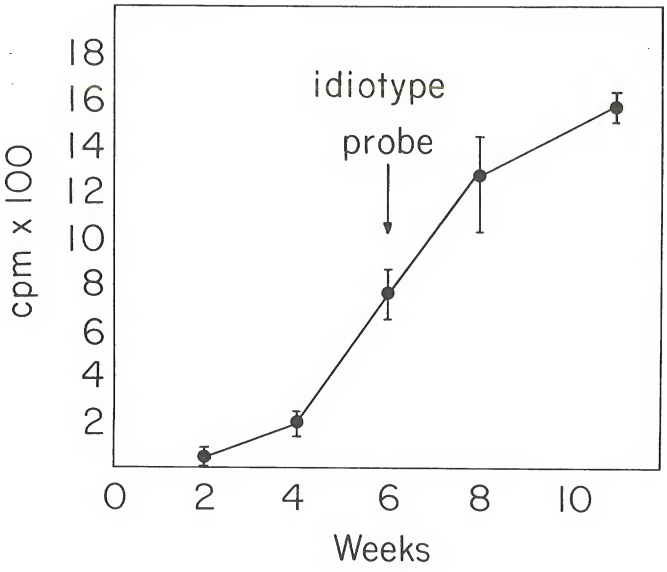
6
8
10
Weeks

Figure 15.

The identification of anti-id in various samples over time, in dog 1, as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and autologous anti-DNP antibody in adjuvant at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.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idiotype
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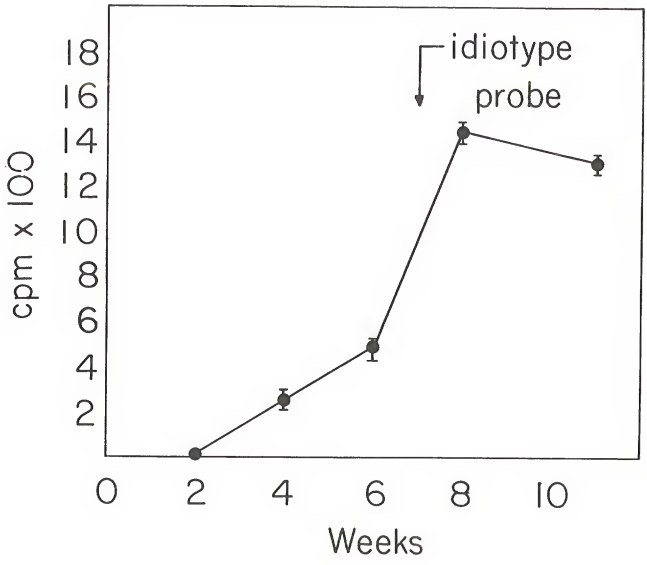
Weeks

Figure 16.

The identification of anti-id in various serum samples, in dog 1, as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and autologous antibody in adjuvant at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 came. These were used as antigens to detect anti-id. The bars represent the standard deviations of the mean.


idiotype
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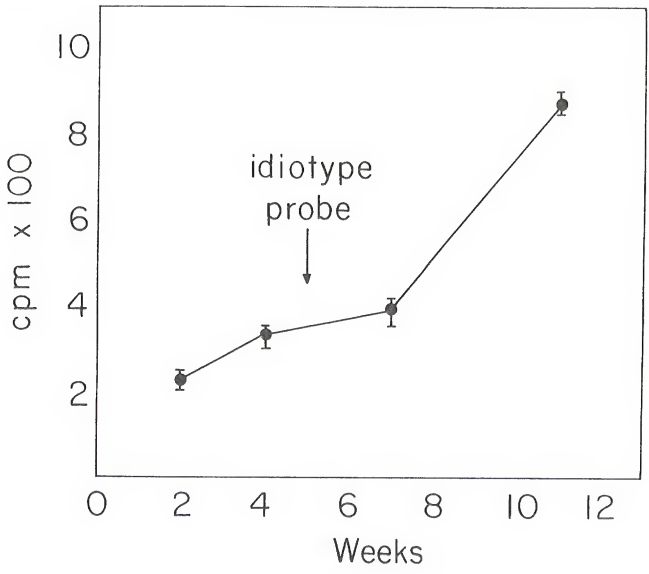
8
10
Weeks

Figure 17.

The identification of anti-id in various serum samples over time, in dog 14, as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and autologous anti-DNP antibody in adjuvant at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.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cpm x 100
idiotype
probe
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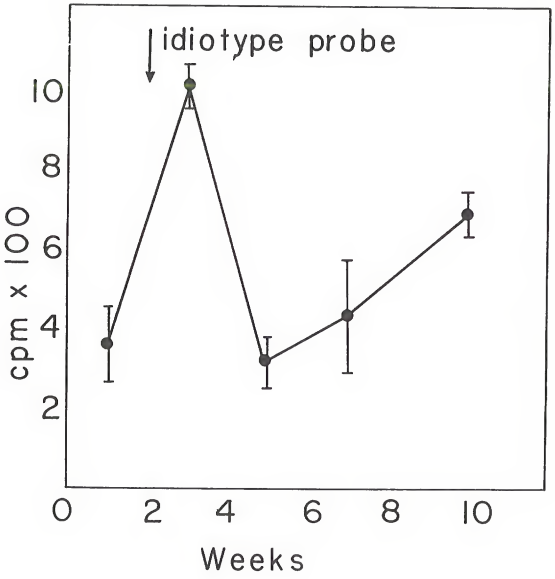
12
Weeks

Figure 18.

The identification of anti-id in various serum samples, as measured in dog 24, by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and CFA at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.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cpm x 100
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6
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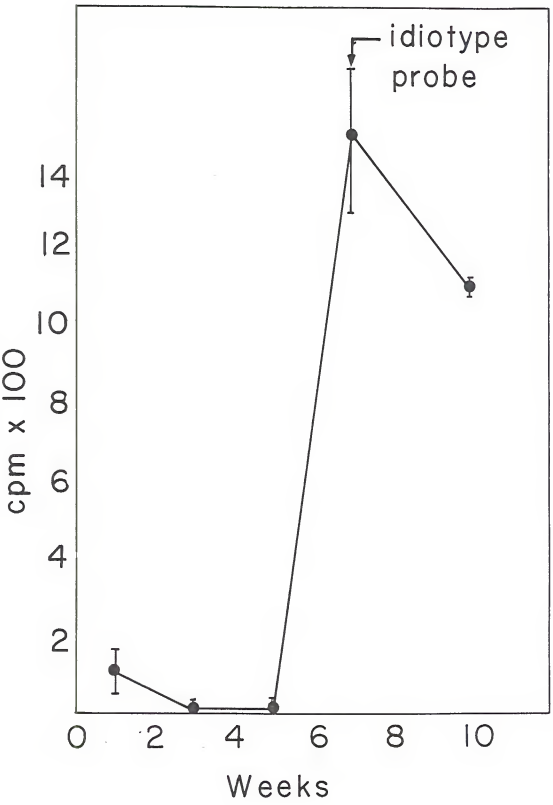
Weeks

Figure 19.

The identification of anti-id in various serum samples over time, in dog 24, as measured by RIA. The dogs received DNP-ASC in adjuvant at weeks 0,2,4,6,8,10, and CFA at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.


idiotype
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cpm x 100
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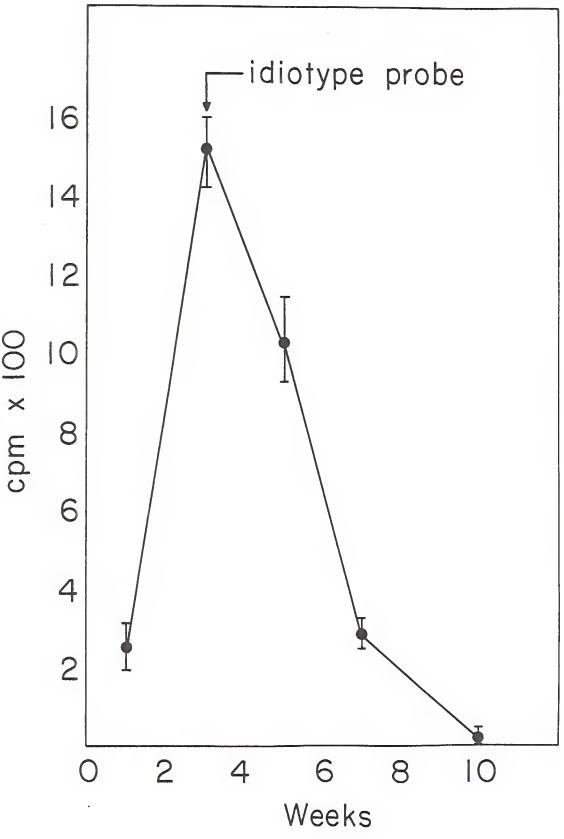
Weeks

Figure 20.

The identification of anti-id in various serum samples, in dog 24, as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8,10, and CFA at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments come. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.


idiotype probe
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cpm x 100
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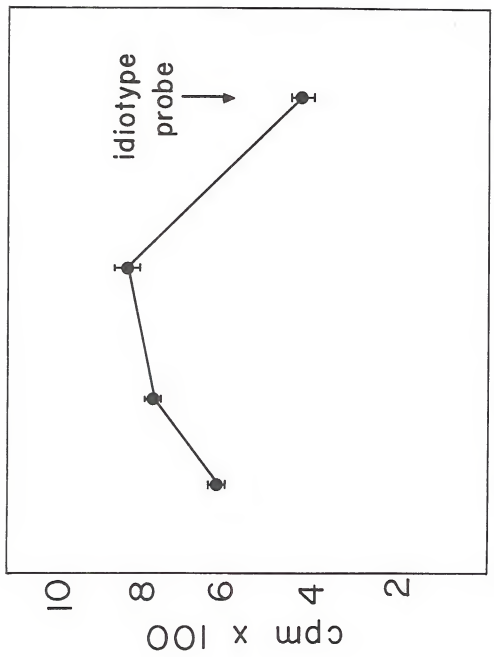
Weeks

Figure 21.

The identification of anti-id in various serum samples, in dog 14, as measured by RIA. THe dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and autologous anti-DNP antibody at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.


idiotype
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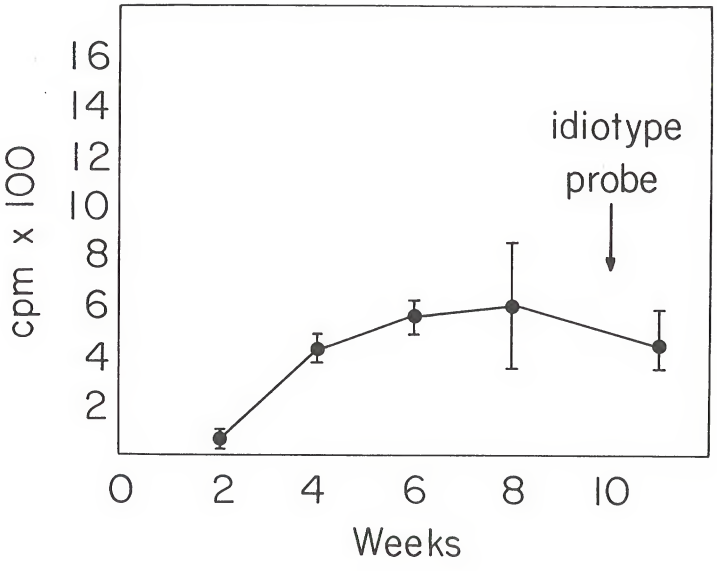
Weeks

Figure 22.

The identification of anti-id in various serum samples, in dog 1, as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and autologous anti-DNP antibody at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.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idiotype
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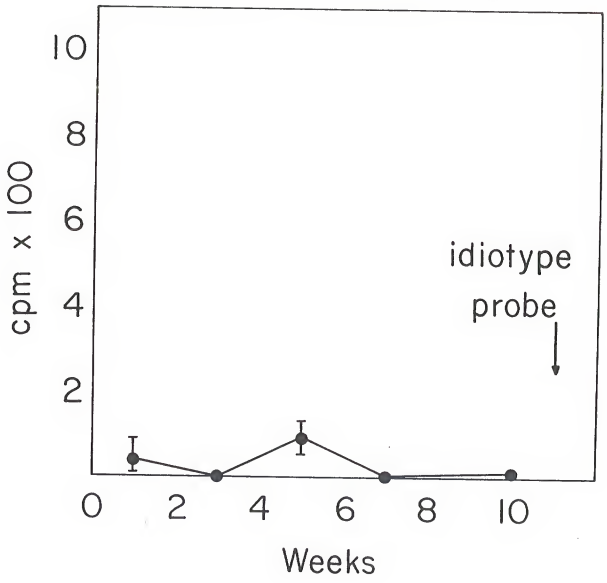
Weeks

Figure 23.

The identification of anti-id in various serum samples, in dog 24, as measured by RIA. The dog received DNP-ASC in adjuvant at weeks 0,2,4,6,8 and 10, and CFA at weeks 7 and 9. The arrow, marked idiotype probe, indicates the time from which the anti-DNP F(ab)'2 fragments came. These were used as antigens to detect anti-id. The bars represent the standard deviation of the mean.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cpm x 100
idiotype
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Weeks

ids obtained from dog 1 at week 10, anti-id was detected in all but the first sample tested (figure 22). Both of these dogs had received autologous antibody in adjuvant. Both these ids were obtained from the dogs after this treatment. In contrast to the anti-id detected in these two dogs, id was obtained from dog 24 at a similar time in the immunization schedule (week 11), but revealed no anti-id using these id antigens. This dog received adjuvant without autologous antibody. In two dogs there was no detectable anti-id at any point in the immunization schedule using autologous id as the probe. This is in spite of the fact that one of the dogs was immunized with autologous antibody and did have detectable levels of anti-id using the mouse monoclonal antibody as the id probe.

Discussion

In the experiments in Chapter four, anti-id was only detected in serum after autologous antibody administration. If anti-id does serve in a regulatory fashion, it is not clear why its presence is not detected during the course of the anti-DNP antibody response. This inability to detect anti-id throughout the immunization schedule may be a function of the xenogeneic probe used to detect anti-id or it may be because anti-id is present only after artificial manipulation. Furthermore, if anti-id does regulate the

reciprocal id, then it would be expected that id would be present in the serum before the appearance of anti-id, but after the appearance of anti-id the reciprocal id would disappear. Since a number of different ids were used as the probe, the corresponding anti-id may be detected either slightly before the point in time the id came from, coincident with the anti-id, or considerably later in time than the id. Alternatively, if id/anti-id were complexed then the disruption of these complexes might allow anti-id to be detected.

When autologous anti-DNP $F(ab)'_2$ was used to assay for anti-id, three different patterns in the detection of anti-id were evident. In the first pattern, anti-id was detected after the appearance of id, but not coincident with nor before its appearance. These results suggest that there was a lag phase between the appearance of id and the corresponding anti-id in the serum. In the second pattern, id and anti-id are present within a single serum sample. The maximum level of anti-id was detected in serum at the same time that the id appeared in two cases. Furthermore, anti-id were present very early in the response. Because the dogs in these experiments would still be expected to have colostrum-derived antibody, these anti-id may represent maternal immunoglobulin. Unfortunately, it was not possible to obtain serum from these bitches to determine if they had anti-id present.

Both the first and second pattern of anti-id are consistent with the hypothesis that id is acting as an antigen to induce anti-id. These data are reminiscent of the type of curves seen when one plots the disappearance of antigen as a function of time and superimposes on that curve the appearance of antibody that is specific for the antigen (90). When antigen is first introduced into an animal there is initially a very slow loss of this antigen from the circulation. After a few days, however, there is a precipitous drop in the level of antigens which is the result of antibody production and is called immune elimination. Antibody when first produced is not detected because it is complexed with antigen and removed from the circulation. At a certain point, however, both antibody and antigen will become apparent in a complexed form. The variables that determine this point include the valence of the antigen and its size, the isotype of the antibody, the affinities between the antibody and the antigen, and the efficiency of the reticuloendothelial system in removing these complexes (90,91). The similarity between immune elimination of antigen and the experimental results obtained suggest that id is removed in a fashion analagous with antigen removal. The appearance of anti-id in the third pattern is difficult to explain. In this case, anti-id was present at a time considerably before the appearance of id. That is, in dog 14, anti-id was

detectable in every serum sample checked (figure 21), and in dog 1, anti-id was detectable in all but the very earliest serum sample (figure 21). Both of these dogs had received autologous antibody in adjuvant and the idiotype used as the antigen was obtained from serum after such treatment. In contrast, when idiotypes from a comparable time in the immunization schedule were used as antigen from, dog 24, which received adjuvant without autologous antibody, the unusual appearance of anti-id was not observed. A possible explanation would be that the administration of autologous antibody in adjuvant stimulated the production of antibody having similar idiotypes. That is, anti-DNP antibodies used for immunization were from week six. The ids on this immunizing antibody may have stimulated additional antibody with the same id. Any anti-id which would be produced because of the id of week six might also bind to ids produced from the immunization of the antibody from week six. Therefore, if this were the case, anti-id could be present in serum prior to the sample from which the id was derived. There is experimental precedence for this suggestion (63,64). Forney et al. (63) have shown that mice given hybridoma-derived anti-sheep red blood cell antibody without stimulation with antigen will subsequently produce anti-SRBC antibody of a similar idiotypic specificity to the immunizing antibody. Their interpretation

was that the antibody stimulated the subsequent production of identical or very similar antibody through an id/anti-id interaction . Therefore, this unusual pattern in the appearance of anti-id could be the result of autologous antibody administration. However, the presence of this anti-id may be the result of factors governing the production and detection of anti-id which are unforseen at this time.

In two of the five animals there is complete failure to detect anti-id using autologous antibody in any serum obtained throughout the immunization schedule. There are a number of different possible explanations for this result. Firstly, only anti-id of the IgG class was measured and it is possible that other anti-id isotypes were produced in these two animals. In fact, in a recent study it was observed that in man there was an isotypic shift over time of the anti-id specific for a given set of auto-antibodies (53). Alternately, there may be certain ids which favor the production of the reciprocal anti-ids. In an experiment in outbred rabbits, anti-id production seemed to be associated with the presence of a few ids that favor anti-id production. Those rabbits not expressing such ids failed to produce detectable anti-id antibodies (57). Based on this, it is possible that, in dogs, certain id are especially important for anti-id production and in those dogs not expressing such ids there is a failure to produce

reciprocal anti-id. This lack of anti-id would not necessarily result in abnormal antibody regulation if these animals had an alternate means to accomplish this, such as a T-suppressor cell pathway.

One of these animals had no recognizable anti-id using autologous antibody as the id probe, but did have recognizable anti-id when mouse monoclonal antibody was used as the id probe. There are several possible reasons for this. 1) There are only a few idiotopes present on monoclonal antibodies while in a heterogeneous population of molecules there would be expected to be many idiotypes and therefore even more idiotopes. Therefore, this negative result may be a function of the concentration of id present in the assay system. 2) The purification process may have altered the id on the antibody molecules just enough so that when this antibody was used to immunize a dog, these altered id were able to induce an anti-id response specific for the mouse id. Or 3) the ids detected with the mouse antibody were different than the ids on the canine anti-DNP $F(ab)'_2$.

Summary and Conclusions

In three of five dogs immunized with DNP-ASC, anti-id could be detected using autologous anti-DNP $F(ab)'_2$

fragments as the id. The kinetics in the appearance of this anti-id in relationship to the id suggest that id is acting as an antigen to stimulate a corresponding anti-id response.

CHAPTER SIX
CONCLUSION

Dogs were chosen to study IgE antibody synthesis and regulation because they develop an IgE mediated disease that is very similar to atopic disease of man.

When dogs were immunized with 100 μg of aluminum hydroxide precipitated DNP-ASC by the intraperitoneal route, each dog synthesized anti-DNP IgG, IgE and IgM antibody. There was a difference in the responsiveness to this antigen between individual dogs which most probably reflected the genetic heterogeneity between them. In an attempt to regulate anti-DNP antibody, autologous anti-DNP antibody in adjuvant was administered to these dogs during the ongoing response. Although dogs so treated did not have a difference in the magnitude of the anti-DNP IgE or IgG response, as compared to control dogs who received adjuvant without antibody, these dogs did produce an anti-id which was detected using mouse monoclonal anti-DNP IgG and IgM.

This anti-id was not an internal image of antigen as shown by 1) a failure of these antibodies to bind to each

mouse monoclonal anti-DNP antibody tested and 2) a failure of hapten to inhibit the id/anti-id interaction. An internal image of antigen should bind to corresponding antibody to an extent similar to antigen and should also be hapten inhibitable (91).

Anti-idiotypic antibody could be detected during the DNP-ASC immune response when the ids used to assay for antibody were autologous anti-DNP $F(ab)'_2$ fragments. These anti-ids can be considered natural in that their appearance was associated with anti-DNP antibody produced during an immune response. This is in contrast to the anti-ids detected with the mouse antibodies. These latter anti-ids were detected subsequent to the immunization with anti-DNP antibody in complete Freund's adjuvant. Three of the five dogs assayed for anti-id using autologous ids had detectable amounts of anti-id whereas two dogs had no detectable levels. It was concluded that anti-id can be detected during a DNP-ASC response in dogs.

This method of detecting anti-id would allow for the identification of a different anti-id response during other immune responses. For example, the aim of hyposensitization for allergic disease is felt to be the production of a blocking antibody. However, this treatment is not always effective, even if blocking antibody is produced. An additional possible reason why hyposensitization works

is because IgE antibody is regulated by id/anti-id interaction. If this is the case, the identification of anti-id during hyposensitization may help in establishing more effective immunotherapy for allergies.

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BIOGRAPHICAL SKETCH

My name is Tippy Schultz. I am a border collie, spitz dog who was an abused puppy scheduled to be euthanized on the day I met my future owner, Kevin Schultz. He rescued me from my fate and since he was in his first year of veterinary school at Purdue University, I became his living anatomical model to study dog topography. I learned that he had been born in Chicago in 1951, and, because his family moved alot, he attended many different grade and high schools before finally moving to Fort Wayne, Indiana, where he graduated from high school. We studied hard in veterinary school and I sent Kevin to work at B.F. Goodrich Tire Company during summers to keep me in food and shelter which provided motivation for him to continue his education.

We graduated in 1976 and since that time, I have gotten to do a great deal of traveling with him. In Dodge City, Kansas, while he was in practice, he met a very pretty and very nice woman. He saved her cat from dying after being hit by a car, and since she could also type, they got married and we all moved to Chicago so Kevin could practice

for a year. We then hit the road for Philadelphia, Pa., where Kevin studied comparative dermatology at the University of Pennsylvania, and he put Nancy to work typing. In September, 1979, we packed up and moved again, this time to Gainesville, Florida. Here both Kevin and Nancy attended the University of Florida and I didn't see much of either of them while they were busy getting their degrees.

During our treks across the country, we have adopted two permanent house guests, Fanny and Pippin, who are OK for being just cats. In July, 1983, we will all be moving again, this time to Boston, Massachusetts. There Kevin will be a post-doctoral fellow in the Department of Pathology at Harvard Medical School which is fine with me as long as he continues to keep me warm and fed.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Richard E.W. Halliwell, Chairman
Professor of Immunology and
Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

George E. Gifford
Professor of Immunology and
Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Parker A. Small, Jr.
Professor of Immunology and
Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Richard B. Crandall
Professor of Immunology and
Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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Immunology and Medical
Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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